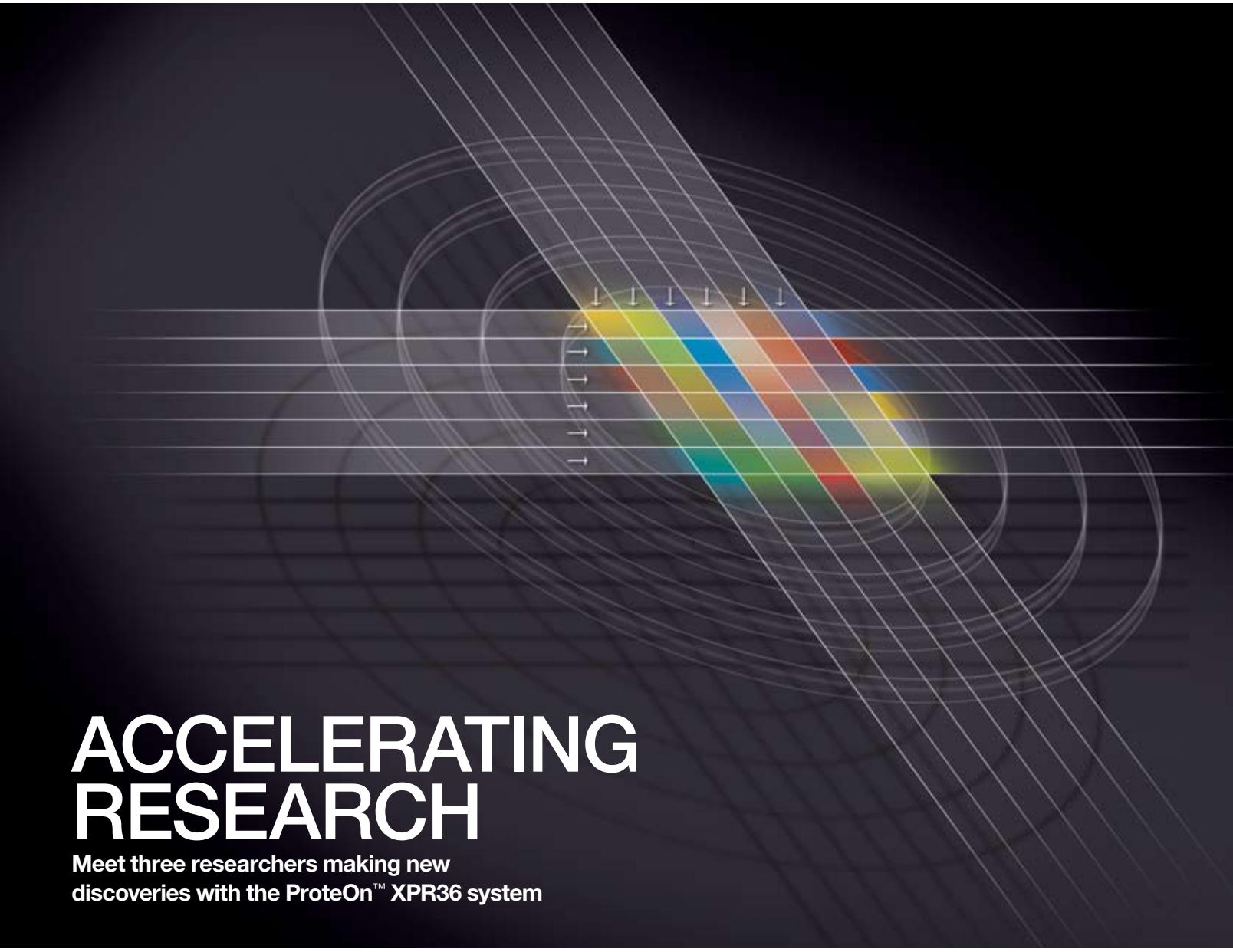
122

BioRadiations

A Resource for Life Science Research



ACCELERATING RESEARCH

Meet three researchers making new discoveries with the ProteOn™ XPR36 system

In this issue:

BIO-RAD

Bio-Plex, Leading Life Science Discovery

Advance your understanding of cell biology and disease mechanisms with the integrated Bio-Plex suspension array system.

The Bio-Plex system is the most widely cited multiplex bead-based assay platform, with research applications in Alzheimer's and Parkinson's diseases, diabetes, obesity, cancer, asthma, cystic fibrosis, autoimmune disease, viral infections, and vaccine development.

The Bio-Plex system enables you to quantitate up to 100 different analytes in one sample with unsurpassed sensitivity, precision, and accuracy, giving you the power to understand complex relationships between proteins in normal and disease states.

For more information, visit us on the Web at **www.bio-rad.com/bio-plex/**

Newest Solution

The Bio-Plex® Precision Pro™ human cytokine 10-plex assay panel.

- Highly sensitive, highly reproducible magnetic bead-based assay
- Optimized for serum and plasma samples
- Detects IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IFN-γ, TNF-α

The Bio-Plex suspension array system includes fluorescently labeled microspheres and instrumentation licensed to Bio-Rad Laboratories, Inc. by the Luminex Corporation.

BIO-RAD SUBSIDIARY
TELEPHONE NUMBERS

Australia 61-2-9914-2800
Austria 43-1-877-89-01
Belgium 32-9-385-55-11
Brazil 55-21-3237-9400
Canada 905-712-2771
China 86-21-6426-0808
Czech Republic 420-241-430-532
Denmark 45-44-52-10-00
Finland 358-9-804-22-00
France 33-1-47-95-60-00
Germany 49-89-318-84-0
Hong Kong 852-2-789-3300
Hungary 36-1-455-8800
India 91-124-402-9300
Israel 03-963-6050
Italy 39-02-216091
Japan 81-3-5811-6270
Korea 82-2-3473-4460
Mexico 55-52-00-05-20
The Netherlands 31-318-540666
New Zealand 64-9-415-2280
Norway 47-23-38-41-30
Poland 48-22-331-99-99
Portugal 351-21-472-7700
Russia 7-095-721-14-04
Singapore 65-6415-3188
South Africa 27-861-246-723
Spain 34-91-590-5200
Sweden 46-8-555-12700
Switzerland 41-61-717-9555
Taiwan 88-62-2578-7189
Thailand 662-651-8311
United Kingdom 44-20-8328-2000
USA Toll free 1-800-4BIORAD
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BioRadiations

issue 122, 2007

TO OUR READERS

Protein-protein interactions drive living systems. Until recently, however, methodical analysis of these complex interactions has been hindered by lack of an affordable, intuitive device able to measure large numbers of interactions in a reasonable amount of time. The ProteOn™ XPR36 protein interaction array system is a surface plasmon resonance (SPR) optical biosensor that can analyze up to 36 protein-protein interactions in a single real-time, label-free experiment. Throughput can reach up to 180 interactions per hour with nanomolar sensitivity. In our feature story, we describe how three life scientists have accelerated and redefined their research using the ProteOn XPR36 protein interaction array system.

COVER STORY

DEPARTMENTS

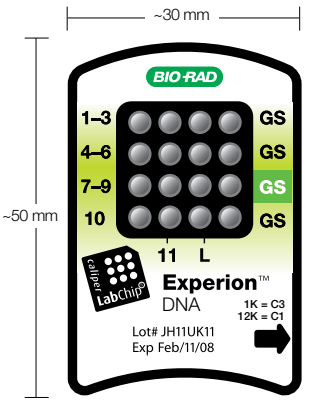
TECHNICAL REPORTS

Legal Notices — See page 32.

Experion™ DNA 1K and DNA 12K Analysis Kits

Experion DNA analysis kits enable automated electrophoresis of DNA on the Experion system. Like the Experion RNA and protein analysis kits, these kits combine innovative chip design with high-quality reagents to perform reproducible, quantitative, and accurate analysis in minutes. Streamlined chip preparation methods and low sample and reagent volume requirements result in rapid experiments with minimal hands-on time.

The Experion DNA 1K and 12K analysis kits allow analysis of DNA samples with size ranges of 25–1,000 bp and 100–12,000 bp, respectively. These DNA assays provide high sensitivity and excellent resolution over a broad dynamic range. Because only 1 µl of sample is needed for each analysis, and the Experion automated system can analyze 11 samples in approximately 30–40 minutes, these assays are great for routine analyses and for applications such as analysis of restriction digests, amplified DNA, microsatellites, and amplified fragment length polymorphisms (AFLPs).



Experion DNA chip. This chip can be used for analysis of up to 11 samples. The DNA 1K and DNA 12K analysis kits use the same chip.

Specifications

	DNA 1K Assay	DNA 12K Assay
Sizing range	25–1,000 bp	100–12,000 bp
Sizing accuracy	±10%	±15%
Number of samples	11	11
Sample volume	1 µl	1 µl
Shelf life	9 months	9 months
Storage conditions		
Reagents	4ºC	4ºC
Chips	Ambient	Ambient

Ordering Information

Catalog #	Description
700-7107	Experion DNA 1K Analysis Kit for 10 Chips, includes 10 DNA chips, Experion DNA 1K reagents and supplies for 10 chips
700-7108	Experion DNA 12K Analysis Kit for 10 Chips, includes 10 DNA chips, Experion DNA 12K reagents and supplies for 10 chips
700-7163	Experion DNA Chips, 10, for DNA 1K and 12K analyses
700-7164	Experion DNA 1K Reagents and Supplies, for 10 chips, includes 3 x 250 µl DNA 1K gel, 40 µl DNA stain, 20 µl DNA 1K ladder, 750 µl DNA 1K loading buffer, 3 spin filters
700-7165	Experion DNA 12K Reagents and Supplies, for 10 chips, includes 650 µl DNA 12K gel, 40 µl DNA stain, 20 µl DNA 12K ladder, 750 µl DNA 12K loading buffer, 3 spin filters
700-7261	Experion DNA 1K Ladder
700-7262	Experion DNA 12K Ladder

Experion™ Software 2.1 Released

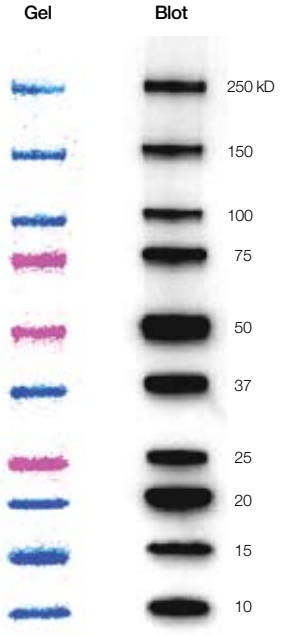
A new version of Experion software is available for the Experion automated electrophoresis system. Assays performed using the DNA 1K and DNA 12K analysis kits require the Experion software version 2.1 update to perform automatic calculations and assess DNA sample results. For a free software upgrade, current Experion system customers can contact Technical Support at 1-800-4BIORAD.

For more information, go to **www.bio-rad.com/ad/experion/**

Ordering Information

Catalog #	Description
700-7050	Experion Software, version 2.1, system operation and standard data analysis tools, PC

Precision Plus Protein™ WesternC™ Standards



Versatile Precision Plus Protein WesternC standards combine the convenience of prestained bands with western blot detection capability. Like other Precision Plus Protein standards, which are designed for applications that require easy and accurate molecular weight estimation, these recombinant protein standards have sharp, accurate, and reproducible bands.

Precision Plus Protein WesternC standards offer ten prestained protein bands over the same broad range as all Precision Plus Protein standards, from 10 to 250 kD. The 25, 50, and 75 kD standards are pink for easy band reference and blot orientation. Each standard has an integral *Strep*-tag sequence to enable parallel detection on western blots using StrepTactin-HRP conjugate. This results in identical band pattern throughout the entire gel electrophoresis and western blotting experiment.

Compatible with film and CCD imagers, such as the Molecular Imager® VersaDoc™ MP and ChemiDoc™ XRS systems, Precision Plus Protein WesternC standards are ideal for visualization of blots, production of publication-quality images of bands on blots, and accurate molecular weight estimation directly from acquired blot images.

Application	Use
Gel electrophoresis	Monitoring migration during electrophoresis
Western blotting	Assessing transfer efficiency
Blot development	Estimating molecular weight using film or CCD imagers

Ordering Information

Catalog #	Description
161-0376	Precision Plus Protein WesternC Standards, 250 µl
161-0380	Precision Protein™ StrepTactin-HRP Conjugate, 300 µl, 150 applications
170-5044	Immun-Star™ GAM-HRP Detection Kit
170-5045	Immun-Star GAR-HRP Detection Kit
170-5046	Immun-Star GAR-HRP Conjugate, 2 ml
170-5047	Immun-Star GAM-HRP Conjugate, 2 ml
170-5070	Immun-Star WesternC Kit

Immun-Star™ WesternC™ Chemiluminescent Kit

The Immun-Star WesternC chemiluminescent substrate kit is designed for blot detection using CCD imagers such as Molecular Imager® VersaDoc™ MP and ChemiDoc™ XRS systems. These CCD imagers offer the advantages of instant image manipulation and greater dynamic range than film. The Immun-Star WesternC kit is compatible with all HRP-conjugated secondary antibodies, and allows detection of long-lasting, intense signals that can be captured optimally by the camera.

When Precision Plus Protein™ WesternC™ standards and sample proteins are transferred to a blot, the standards can be displayed on the same acquired image, offering unparalleled accuracy in molecular weight estimation.

Advantages of the kit include:

- Optimization for CCD imagers — for image-based qualitative and quantitative analysis of western blots
- Strong signals that last for 24 hours — enables multiple exposures for image optimization
- Great sensitivity — mid-femtogram (10^{-14}) detection
- Long-term storage — the kit is stable for at least one year at room temperature; the working solution is stable for 24 hours at room temperature

Ordering Information

Catalog #	Description
170-5070	Immun-Star WesternC Kit, includes 50 ml luminol/enhancer solution and 50 ml peroxide buffer
161-0376	Precision Plus Protein WesternC Standards, 250 µl
170-8650	Molecular Imager VersaDoc MP 5000 System, PC or Mac
170-8070	Molecular Imager ChemiDoc XRS System, PC
170-5046	Immun-Star GAR-HRP Conjugate, 2 ml
170-5047	Immun-Star GAM-HRP Conjugate, 2 ml

MicroRotofor™ Cell Lysis Kits

The MicroRotofor cell lysis kits can be used to extract proteins from small amounts of tissue and to prepare samples for the MicroRotofor cell, which performs liquid-phase isoelectric focusing (IEF) in only 2.5 ml. This effective method for reducing sample complexity allows visualization of low-abundance proteins in downstream applications.

The kits are convenient and easy to use, and provide tailored protocols based on sample type. All kits are quality controlled to guarantee performance and help ensure reproducibility of IEF runs.

- Cell lysis and extraction protocols are tailored for mammalian, plant, yeast, and bacterial samples
- Resulting protein extract can be run immediately in the MicroRotofor cell — no need to change buffer conditions to accommodate the run
- Kits can be used for any species-specific protein extraction — a MicroRotofor run is not a necessary downstream application

For more information on the MicroRotofor cell, go to **www.bio-rad.com/microrotofor/**

Ordering Information

Catalog #	Description
163-2141	MicroRotofor Cell Lysis Kit (Mammal), 15 preps, 50 ml protein solubilization buffer (PSB), ReadyPrep™ mini grinders (2 packs of 10 each)
163-2142	MicroRotofor Cell Lysis Kit (Plant), 10 preps, 50 ml protein solubilization buffer (PSB), ReadyPrep 2-D cleanup kit (50 reaction size)
163-2143	MicroRotofor Cell Lysis Kit (Yeast), 15 preps, 50 ml protein solubilization buffer (PSB), 15 ml yeast suspension buffer, 2 x 0.5 ml lyticase (1.5 U/µl)
163-2144	MicroRotofor Cell Lysis Kit (Bacteria), 15 preps, 50 ml protein solubilization buffer (PSB), 25 ml bacteria suspension buffer, 1 ml lysozyme (1,500 U/µl)

siLentMer™ Validated Dicer-Substrate siRNA Duplexes

siLentMer Dicer-substrate siRNA duplexes for silencing of the gene targets listed below are now available. These targets cover a variety of research interests and significance, including genes involved in tumor suppression, cell adhesion and motility, inflammation, cell signaling, and many diseases and disorders, including Alzheimer's, rheumatoid arthritis, respiratory disease, autoimmune disorders, and peripheral vascular disease. For more information, go to **www.bio-rad.com/RNAi/**

Ordering Information

Genes of Research Interest*

Gene Target	Duplex 1, Catalog #	Duplex 2, Catalog #	NCBI Accession #
Human CDK5	179-0120	179-0220	NM_004935
Human ILK	179-0121	179-0221	NM_001014794
Human CHUK	179-0122	179-0222	NM_001278
Human MAPK8	179-0123	179-0223	NM_002750
Human BAX	179-0124	179-0224	NM_004324
Human MAP2K1	179-0125	179-0225	NM_002755
Human BCR	179-0126	179-0226	NM_004327
Human BRCA1	179-0127	179-0227	NM_007294
Human PTK2	179-0128	179-0228	NM_005607
Human STAT1	179-0129	179-0229	NM_007315
Human ATF2	179-0130	179-0230	NM_001880
Human ELK1	179-0131	179-0231	NM_005229
Human RB1	179-0132	179-0232	NM_000321

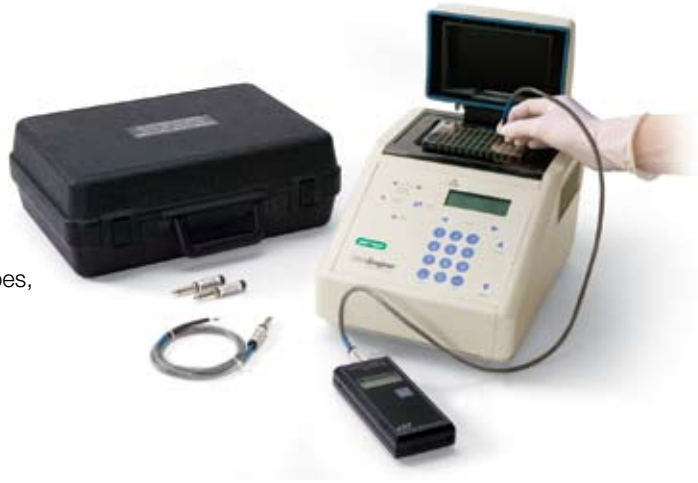
Catalog #	Description
179-0000**	siLentMer siRNA Resuspension Buffer, 1.0 ml
179-0001**	siLentMer Fluorescently Labeled Nonsilencing siRNA, 1 nmol, control for delivery
179-0002**	siLentMer Nonsilencing siRNA, 1 nmol, negative control for silencing (unlabeled)

* siLentMer Validated Dicer-Substrate siRNA Duplexes, 2 nmol, designed with proven criteria and functionally tested for >85% silencing.
** Kit components offered separately.

Verification Probe™ Thermometer

The Verification Probe thermometer now enables field evaluation of all Bio-Rad thermal cyclers and blocks, including the DNA Engine® family, PTC-100®, MyCycler™, iCycler®, and MJ Mini™ thermal cyclers. The Verification Probe thermometer operates independently of the thermal cycler, which is important in laboratories where CAP or NCCLP regulations require periodic validation procedures. These verification procedures can also be important in high-throughput laboratories where it is important to verify that a large number of thermal cyclers are functioning equivalently.

Separate probes are available to fit sample blocks for 0.5 ml tubes, 96-well plates/0.2 ml tubes, or 384-well plates. Probes are sold as separate kits, and periodic verification of function may be required.

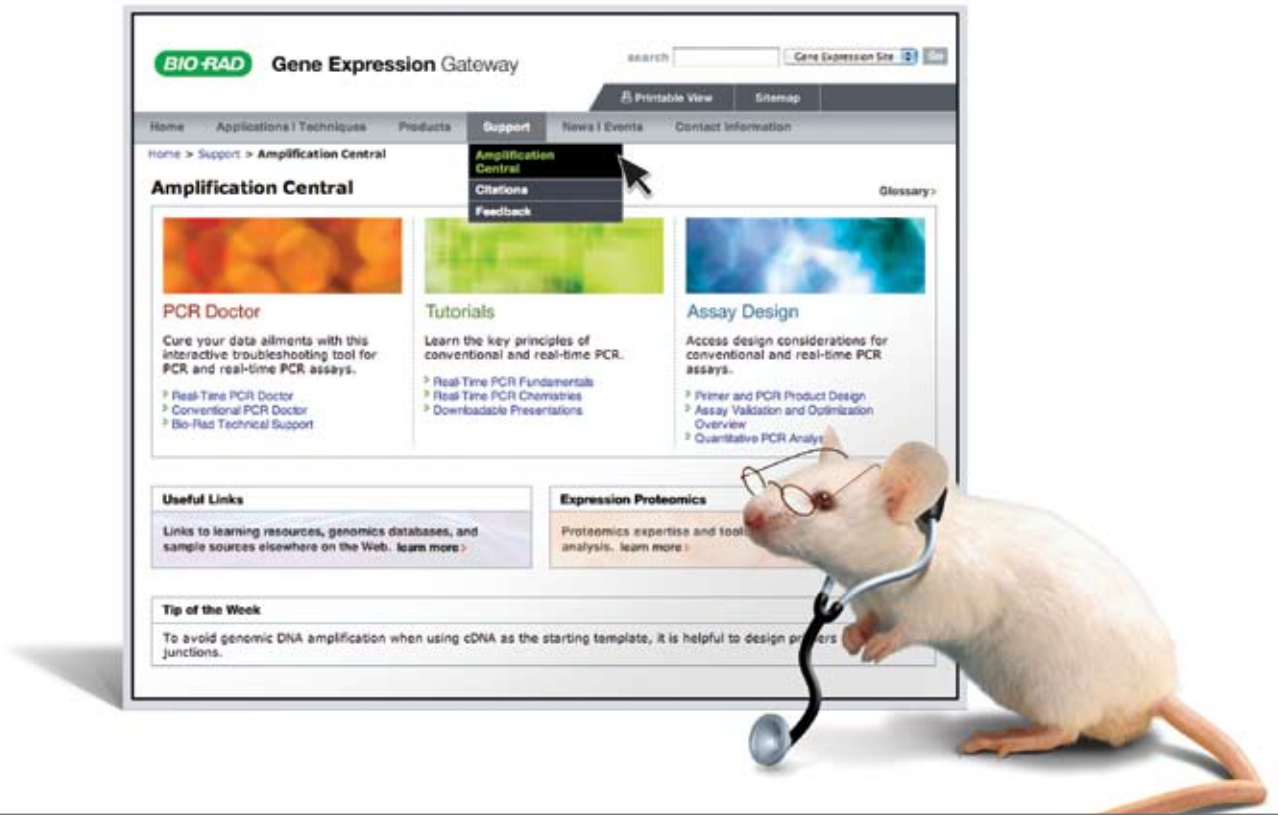


Key Features

- Handheld digital thermometer with 0.1°C resolution
- Laser-trimmed probes to fit precisely in Bio-Rad thermal cyclers
- NIST-traceable — accuracy of both probe and thermometer to ±0.4°C at 60°C and 90°C
- Probe placement templates to guide probe insertion, minimize air currents, and ensure reproducible test results
- Detailed test protocols for all of the thermal cyclers mentioned above

Ordering Information

Catalog #	Description
VPT-0300	Verification Probe Thermometer, NIST-traceable, 0–100°C range, ±0.4°C accuracy at 60°C and 90°C; order with appropriate probe(s)
VPK-0005	Probe Kit, for VPT-0300, fits 0.5 ml blocks, NIST-traceable
VPK-0002	Probe Kit, for VPT-0300, fits 0.2 ml blocks, NIST-traceable
VPK-0384	Probe Kit, for VPT-0300, fits 384-well blocks, NIST-traceable

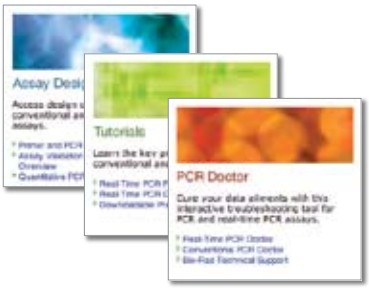


Amplification Central — Just what the PCR Doctor ordered.

Now there are even more reasons to mouse on over to Bio-Rad's Gene Expression Gateway:

- Educational tutorials and slide presentations
- Troubleshooting and solutions for problems commonly found in PCR data
- Guidelines for experimental design, optimization, and validation
- Weekly tips for improving PCR design and laboratory practices
- Abstracts of the most-cited PCR and real-time PCR articles

Your gateway to Bio-Rad's extensive genomics expertise is just a click away. Visit us on the Web at **www.bio-rad.com/genomics/pcrsupport/**



PCR Doctor, assay designs, and a host of guided tutorials will help you achieve successful research results.

Practice of the polymerase chain reaction (PCR) may require a license.

Humidification of the Slide Chambers™ Dual Alpha™ Unit for Precise Temperature Control of Slides

Introduction

In situ thermal cycling on glass slides offers great potential for the detection and localization of viral infections, the investigation of developmental and tissue-specific gene expression, and monitoring the results of gene therapy. This body of techniques includes a constellation of related methods, including in situ PCR, in situ RT-PCR, and in situ hybridization. The success and reproducibility of these techniques depend critically on the precision and uniformity of the slide thermal cycler and on preventing slides from drying out during the cycling process. A vapor-tight reaction chamber is needed for slides undergoing thermal cycling, but for hybridizations, a semi-open reaction chamber (for example, a slide with coverslip or a Hyb-Seal™ chamber) is a convenient option so long as the incubation chamber can be humidified.

The Slide Chambers Dual Alpha unit is a compact system that is well suited for slide-based hybridizations as well as slide thermal cycling for up to 30 slides. The accurate and uniform temperature control (±0.6°C) of the Peltier effect-based reaction module ensures highly reproducible reaction conditions across each slide and from slide to slide. The following procedure can maintain a humid environment in the Slide Chambers unit for 24 hours with a simple humidification pad. By adding flared ends to the pad (Figure 1), a humid chamber can be maintained for over 2 days.

Hyb-Seal chambers (Figure 2) can be used to create a vapor-tight chamber on a slide for thermal cycling reactions. For humidified hybridization steps in the workflow, the injection ports can be left uncovered to simplify sample handling steps.

Preparing the Humidification Pad

1. Cut a sheet of extra thick blotting paper (for example, Bio-Rad catalog #170-3960) into 9.2 x 2.5 cm pads or pads with flared ends, as in the template shown in Figure 1.
2. Partially insert the unflared end of one pad into the lowest slot of the slide tower.
3. Using a transfer pipet, slowly add 3–4 ml of water to the pad until the pad is saturated (Figure 3).

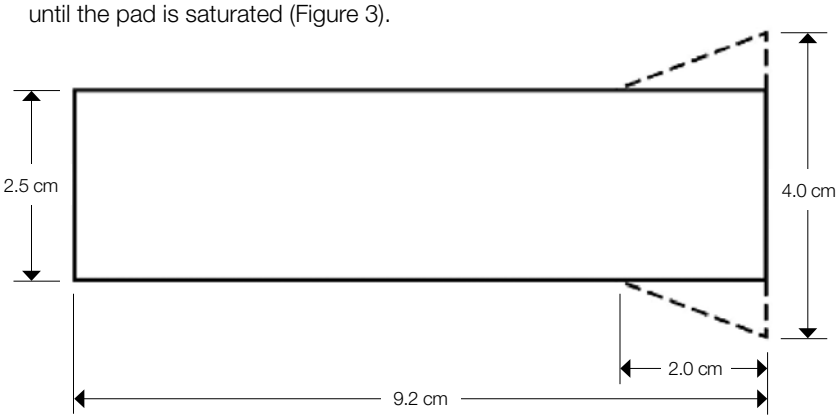


Fig. 1. Template for creating the humidification pad. (Actual size is shown.)

Fig. 2. Hyb-Seal chambers for slide-based temperature incubations.

4. Fully insert the pad into the slot until it touches the rear panel, then lightly bend the front of the pad down so that it touches the base of the tower. When the door is closed, the pad should touch the clear acrylic window. The pad collects and recirculates any condensation that runs down the window.

Preheating and Humidifying the Slide Chambers Dual Alpha Unit

For a procedure that requires an initial denaturation step, such as a hybridization protocol, prepare the humidification pad as described, then follow these steps:

1. Program the Slide Chambers unit with three incubation steps: the first at the desired denaturation temperature (for example, 95°C) for "forever", the second with the desired denaturation temperature and time (for example, 2 min), and the third with the appropriate incubation temperature and time.
2. While the slides are being readied, start the protocol (chamber doors closed) and allow the Slide Chambers unit to hold at 95°C until the chamber is fully humidified (about 2–3 min).
3. When the slides are ready, insert them into the Slide Chambers unit, close the door, and press Proceed. This advances the program to the second step and begins the denaturation and incubation sequence according to the programmed conditions.

Fig. 3. Saturating the humidification pad with water.

Validation Process for siLentMer™ Dicer-Substrate siRNA Duplexes

Introduction

Small interfering RNAs (siRNAs) are widely and increasingly used to silence genes of interest. Upon entry into the cell, these powerful double-stranded RNA molecules initiate RNA interference (RNAi) mechanisms by targeting specific, homologous mRNA sequences. These mechanisms result in cleavage of mRNA molecules that are complementary to the introduced siRNA. Once a large portion of the targeted mRNA molecule is recognized and cleaved, the corresponding gene is effectively silenced. For an excellent review of RNAi, see Tomari and Zamore (2005).

Before the discovery of RNAi, studying specific genes in mammalian cell lines was typically difficult, time-consuming, and very costly. Often researchers had to derive their cell line from a knockout mouse, or were limited by associating mutations with obvious organism phenotypes, or required specific chemical inhibitors that could inhibit one but usually not all enzyme functions. Now, by harnessing the RNAi mechanism, researchers are able to silence any expressed gene for which an effective siRNA sequence can be designed. Compared to previous methods of studying gene function, RNAi is cost-effective, flexible, and easy to perform.

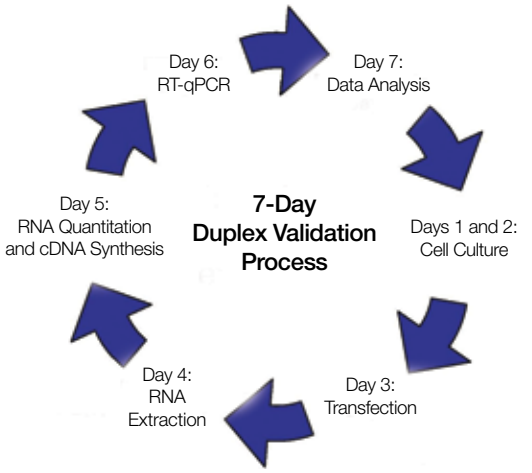
Key elements of a successful RNAi experiment are good transfection efficiency and the use of an effective siRNA. The method of delivery, whether via lipid reagents, viral vectors, or electroporation, should be optimized before attempting to silence a gene of interest. The effectiveness of siRNA delivery can be determined by delivering a fluorescently labeled siRNA and visually evaluating delivery, or by delivering an siRNA to a reference or housekeeping gene, such as GAPDH or β-actin, of known effectiveness and assessing the expression results.

Once the delivery method is established and validated, the success of an RNAi experiment will depend on the potency of the siRNA used. Recent findings suggest that increasing the length and altering the end structure of double-stranded RNAs makes them more potent gene silencing molecules than traditional siRNAs (Kim et al. 2005, Rose et al. 2005). In addition, these enhanced RNA molecules, 27 nucleotides long, are capable of maintaining gene silencing for a greater duration, up to 6 days longer, than traditional siRNAs (Kim et al. 2005). The key to this improved potency and longevity is likely an enhanced and controlled interaction with the RNA endonuclease Dicer (Kim et al. 2005, Rose et al. 2005). Therefore, siRNAs incorporating this improved design strategy are called Dicer-substrate siRNAs.

Bio-Rad offers siLentMer Dicer-substrate siRNA duplexes and validates the performance of many of these siRNAs to ensure >85% knockdown of the target mRNA. This article describes the experimental procedure used to validate siLentMer siRNA duplexes and outlines the criteria that a siLentMer siRNA duplex must meet before it is offered as a validated duplex. The procedure described can be applied by anyone performing optimization experiments for any duplex RNA.

Validation Process

The performance of siLentMer siRNA duplexes is validated in HeLa cells grown under optimized conditions (see figure). Before siRNA delivery, HeLa cell cultures are reinoculated twice in 2 days to ensure that the cells will be in log phase growth at the time of transfection. Bio-Rad's siLentFect™ lipid reagent, which was designed specifically for cellular delivery of small RNA molecules, is used to deliver siLentMer siRNAs into HeLa cells. The cells are transfected with a siLentMer nonsilencing negative control RNA or a siLentMer siRNA specific to the gene of interest, both at a final concentration of 5 nM. After siRNA delivery, cells are incubated for 24 hr before RNA is extracted. RNA extraction is followed by spectrophotometric quantitation and cDNA synthesis using a Bio-Rad iScript™ cDNA synthesis kit. Prior to cDNA synthesis, RNA quality is assayed in random samples, which can be performed using an Experion™ automated electrophoresis system. The silencing efficacy of the siLentMer duplex is measured by RT-qPCR. The level of gene-specific amplification in the negative control is compared to that in the test duplex, and the percent silencing is calculated. The percentage value determines whether a particular siLentMer siRNA duplex is validated or discarded.



Experimental workflow for siLentMer duplex validation. HeLa cells are cultured, then transfected in two 48-well plates with siLentFect lipid reagent and 5 nM siLentMer Dicer-substrate siRNA duplexes. RNA is extracted and quantitated by spectrophotometry, and cDNA synthesis is performed using the iScript cDNA synthesis kit. Final steps include RT-qPCR and data analysis using the iQ™5 real-time PCR detection system and Excel software.

siLentMer Pass/Fail Criteria and Quality Control

To become validated, siLentMer siRNAs must pass two rounds of testing. In the first round, a single validation test is performed for each siLentMer duplex targeting a gene of interest. The siLentMer siRNAs that silence the gene of interest by at least 80% are subjected to a second round of testing. In the second round, each siLentMer duplex is tested in duplicate. To pass the second round of testing, a siLentMer duplex must silence the gene of interest by at least 80%. The data from both rounds (three replicate tests) are combined, and the average percent silencing and standard deviation are calculated. To pass this analysis and become a validated siLentMer duplex, the average percent silencing must be ≥85% and the standard deviation must be <2.5%.

In addition to the rigorous validation procedure that siLentMer siRNAs undergo, every siLentMer duplex sold by Bio-Rad is measured by HPLC to ensure that >85% of the RNA molecules are in duplex form. Furthermore, the duplexes are analyzed by mass spectroscopy to guarantee that the correct sequence was synthesized.

References

Kim DH et al., Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy, Nat Biotechnol 23, 222–226 (2005)

Rose SD et al., Functional polarity is introduced by Dicer processing of short substrate RNAs, Nucleic Acids Res 33, 4140–4156 (2005)

Tomari Y and Zamore PD, Perspective: machines for RNAi, Genes Dev 19, 517–529 (2005)

Patented Seal in MiniOpticon™ Real-Time System Maximizes Life of Thermal Electric Modules

The patented* O-ring hermetic seal found in MJ Mini™ thermal cyclers and MiniOpticon real-time systems forms an airtight barrier around the thermal electric modules (TEs) of the thermal cycler (see figure). As the heating and cooling elements, TEs are the hardest-working components of thermal cyclers, and could be subject to degradation due to condensation from constant heating and cooling. The patented O-ring gasket prevents moisture from entering the TEs, substantially lengthening their life and ensuring outstanding thermal performance.

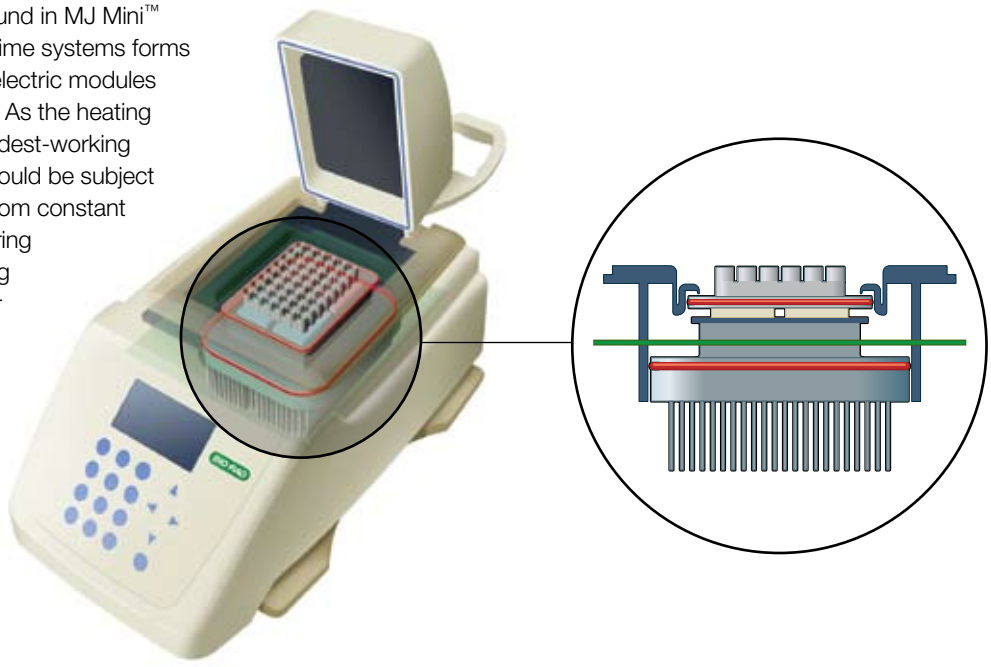


Illustration of MJ Mini reaction module. Upper and lower O-ring gaskets form a hermetic seal around thermal electrics for a dramatic improvement in thermal cycler longevity. Enlarged view shows cross-section of reaction module.

* US patent 7,051,536.

Biomarker Research Centers — Accelerating Biomarker Discovery

Scientific Expertise, Rapid Results

Bio-Rad's Biomarker Research Center facilities offer state-of-the-art proteomics research services, enabling rapid biomarker discovery and assay development in a variety of research areas. These research services enable collaborators to leverage Bio-Rad's internal scientific expertise and emerging technology solutions to answer clinical research questions efficiently and effectively; typically, biomarkers are delivered from biological samples in a matter of weeks.

Biomarker Research Centers offer customized study design and data analysis, robotics to increase throughput, and access to novel methodologies for enriching low-abundance proteins (see sidebar). Our scientists have proven experience with discovery and characterization of biomarkers in many disease areas, including oncology, neurology, and cardiovascular and infectious diseases. They use optimized protocols for analysis of a diverse range of samples, including serum, plasma, urine, cerebrospinal fluid, cell lysates, lavage, and laser-capture microdissected cells. Their experience enables them to provide collaborative support in a wide range of projects, including:

- Disease monitoring (drug efficacy, progression, recurrence)
- Toxicology and drug safety
- Patient stratification
- Assay development

In addition to providing research services, Biomarker Research Centers can provide technology transfer and training support to facilitate implementation of the optimized processes into a collaborator's laboratory.

What Biomarker Research Centers Offer

- Expertise in study design to yield optimum results with the samples available
- Standardized control of instrumentation and data to ensure high-quality results
- Experience with customized biostatistical analysis tools to accommodate complex study designs
- Optimum sensitivity with implementation of the latest developments in surface-enhanced laser desorption/ionization (SELDI) technology
- Rapid results using liquid-handling robotics to increase throughput and reproducibility

Collaborative Research Options

Pilot studies, collaboration programs, and affinity bead low-abundance protein enrichment services are all available through the Biomarker Research Centers.

Pilot studies provide a wide range of options for technology evaluation and preliminary biomarker discovery. Pilot studies include:

- Fractionation of complex samples using anion exchange or affinity bead low-abundance protein enrichment methods
- Profiling under multiple unique conditions, including varying combinations of fractionation methods, array chemistries, and mass optimization ranges to increase the total number of proteins detected
- Customization of study designs to accommodate various budgets and project requirements
- Analysis of 40 or more samples for heterogeneous human population studies, with smaller sample set requirements for preclinical animal models, cell cultures, and tissue samples

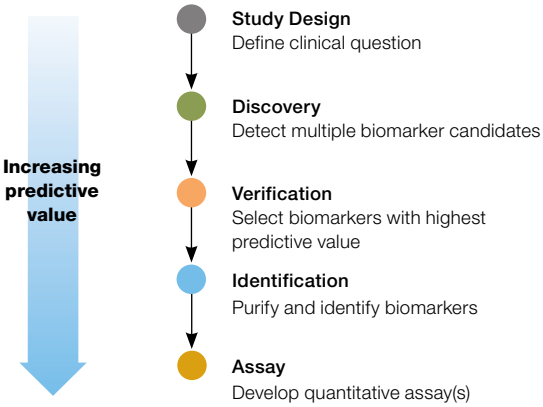
Collaboration programs are designed to provide in-depth biomarker discovery and characterization. Collaboration programs offer:

- Accommodation of large numbers of samples and complex study designs
- Optimized protocols and customized statistical analysis to assess disease state, drug toxicity, efficacy, and drug response profiles
- Protein purification/identification and assay development capabilities
- Technology transfer available at any stage

Affinity bead low-abundance protein enrichment sevices allow samples to be fractionated to enrich low-abundance proteins and dilute high-abundance proteins; fractions are returned to clients for downstream analysis (see sidebar).

Global Capabilities

To learn more about establishing a collaborative research program with one of our Biomarker Research Center facilities, located in Copenhagen, Yokohama, and Philadelphia, please contact your local Bio-Rad representative or e-mail us at BRC@bio-rad.com.



The Biomarker Research Center discovery process.

Low-Abundance Protein Enrichment

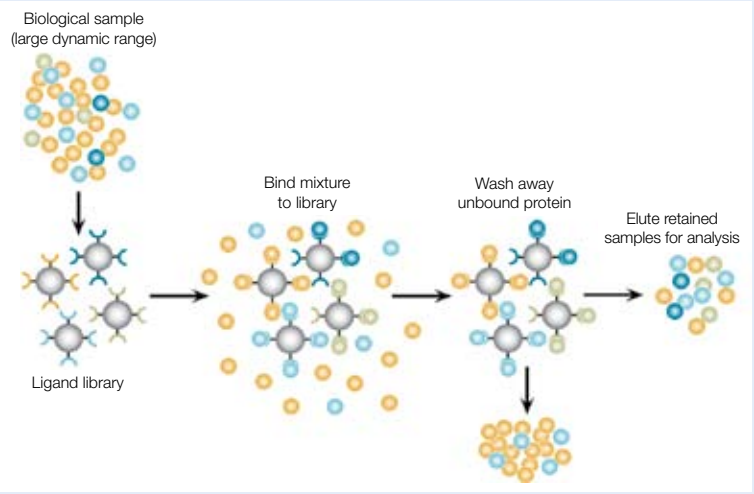
Exploring the Low-Abundance Proteome

Dynamic range issues associated with complex biological samples like sera or plasma make the detection of low-abundance proteins extremely challenging. Current approaches to address this problem focus on lowering the concentrations of high-abundance proteins by immunodepletion methods. Unfortunately, these antibody-based techniques are typically restricted to small sample volumes and can result in dilution of the trace proteins present in a sample.

Affinity Bead Solution

Affinity low-abundance protein enrichment beads represent a novel way to explore low-abundance proteins in biological samples. They simultaneously enrich low-abundance proteins and dilute high-abundance proteins (Castagna et al. 2005, Fortis et al. 2006, Thulasiraman et al. 2005), significantly increasing the number of unique protein species that can be detected (see figure). This technology is compatible with a wide range of sample volumes, from a few hundred microliters to milliliters or liters of starting material (Righetti et al. 2006). The resulting samples provide an opportunity to explore a whole new depth of the proteome.

Access to this affinity bead technology is available through Bio-Rad's Biomarker Research Centers. Samples are treated on a fee-per-sample basis. The Biomarker Research Centers will fractionate samples, typically yielding three or four fractions, which are then returned to collaborators for further analysis using techniques such as 2-D electrophoresis and SELDI. Samples are processed using protocols optimized for the type of sample, including protocols for serum, plasma, urine, cerebrospinal fluid, cell lysates, and lavage.



Affinity bead technology. Each bead features a different hexapeptide ligand with affinity for specific proteins in a sample. When a complex protein sample is incubated with a bead library, protein components find their binding partners. Excess high-abundance proteins will not be captured on binding sites and will be depleted once the beads are washed. In contrast, low-abundance proteins will be concentrated on their specific affinity ligand. In this way, low-abundance proteins are enriched relative to the high-abundance proteins in the sample. No fraction is discarded in this approach, and proteins that might bind to high-abundance proteins like albumin are retained.

If you would like to learn more about how to initiate a low-abundance protein enrichment project with one of our Biomarker Research Center facilities, please contact your local Bio-Rad representative or e-mail us at BRC@bio-rad.com.

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Screening, Ranking, and Epitope Mapping of Anti-Human IL-9 Supernatants

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Introduction

Monoclonal antibodies have become a particularly important resource for medical research, basic science, and disease diagnosis and therapy. Numerous applications utilizing monoclonal antibodies have been developed, including disease diagnosis, drug delivery to target cells (cancer treatment, immune suppression, HIV treatment), biosensors, organic molecule identification, blood clot detection, and septic shock treatment.

The ProteOn™ XPR36 protein interaction array system is a surface plasmon resonance (SPR) instrument with parallel sample processing capabilities that meets the challenge of high throughput while maintaining all the unique qualities of optical biosensors. The ProteOn system uses a novel microfluidics system to perform simultaneous measurements in an array of 36 interaction spots and 42 local reference spots (interspots). All stages of the experiment, from surface immobilization of "ligand" proteins to their subsequent reaction with "analyte" molecules, are measured in real time to provide comprehensive data for 36 interactions.

Here, we demonstrate the application of the ProteOn XPR36 system to the selection, ranking, and epitope mapping of 20 monoclonal antibody supernatants against human interleukin 9 (IL-9). Anti-mouse antibody was immobilized on a chip and used to capture supernatant antibodies that were further reacted with the IL-9 antigen to determine binding kinetic constants. Four antibodies were then purified, their kinetic rate constants were reanalyzed, and epitope mapping was performed to identify antibodies recognizing different epitopes. We capitalized on the throughput provided by ProteOn XPR36 array technology, capturing five different supernatant antibodies in parallel and then determining their binding kinetic constants in a single injection comprising five antigen concentrations. Repeating this cycle four times allowed the determination of the kinetic parameters of all 20 supernatants within 1 hour.

Methods

Instrumentation and Reagents

Experiments were performed using the ProteOn XPR36 system. Phosphate buffered saline with 0.005% Tween 20 (PBS/Tween), pH 7.4 was used as running buffer throughout, and all experiments were performed at 25°C. Recombinant human IL-9 was obtained from PeproTech, Inc. Anti-mouse IgG (whole molecule) was obtained from Sigma, and mouse anti-human IL-9 antibody supernatants were obtained by immunizing BALB/c mice with recombinant human IL-9. Hybridomas were obtained after fusion between spleen lymphocytes and Sp2 myeloma cells.

Screening and Ranking of Anti-Human IL-9 Supernatants

Anti-mouse IgG was immobilized on a ProteOn GLM sensor chip using standard amine coupling chemistry. Using a flow rate of 30 µl/min, the microfluidics network was directed to inject 200 µl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 0.05 M *N*-hydroxysulfosuccinimide (sulfo-NHS) followed by 200 µl of 90 µg/ml of anti-mouse IgG diluted in 10 mM sodium acetate buffer, pH 4.5 (Figure 1A). The surface was then deactivated with 150 µl of 1 M ethanolamine HCl, pH 8.5 and conditioned using 30 µl of 0.85% phosphoric acid, both at a flow rate of 100 µl/min. About 10,000 RU of anti-mouse IgG was immobilized. Next, five different mouse anti-human IL-9 antibodies (in supernatants) were captured by injecting 150 µl of the supernatants into five of the channels containing immobilized anti-mouse IgG (ligand channels) at a flow rate of 25 µl/min (Figure 1B). A sample of running buffer was injected into the sixth channel, which was used as a reference channel.

At the second step of analyte binding, the microfluidics network directed flow into the six analyte channels orthogonal to the ligand channels (Figure 1C). Five human IL-9 concentrations (6.25, 12.5, 25, 50, and 100 nM) were injected (100 µl) at a flow rate of 50 µl/min. The five analyte concentrations reacted simultaneously with each of the five captured anti-human IL-9 antibodies in a single injection. Running buffer was injected into the sixth channel for double referencing to correct the slow dissociation of the captured antibody from the capturing anti-mouse IgG antibody. The antibody/antigen complex was removed from the chip by regeneration with 30 µl of 0.85% phosphoric acid at a flow rate of 100 µl/min, allowing the capture of five more anti-human IL-9 antibodies. This workflow was repeated four times until all 20 supernatants were analyzed.

Determining Kinetic Constants of Purified Anti-Human IL-9 Antibodies

Anti-human IL-9 antibodies showing high affinities were purified by affinity chromatography using a protein A support and then immobilized on a ProteOn GLC sensor chip using the amine coupling chemistry described above (EDAC and sulfo-NHS were

used for the covalent binding of the purified antibodies to the chip surface). The purified antibodies were diluted with sodium acetate buffer, pH 4.5 to a final concentration of 8 µg/ml and injected into four of the flow channels for 4 min at a flow rate of 30 µl/min. The fifth channel was unmodified and served as a reference channel; the sixth channel remained unused. The chip surface was then deactivated using 1 M ethanolamine HCl, pH 8.5. Ligand densities of 1,000–1,200 RU were achieved. Six concentrations (3.18, 6.25, 12.5, 25, 50, and 100 nM) of human IL-9 were injected (75 µl) into the six channels orthogonal to the channels containing immobilized antibodies at a flow rate of 50 µl/min. The data were analyzed and fitted to a simple 1:1 interaction model.

Epitope Mapping of Anti-Human IL-9 Antibodies

Epitope mapping was performed with the same ProteOn GLC sensor chip used for kinetic analysis directly after regeneration, which was performed with 0.85% phosphoric acid (short pulse of 20 sec). A single concentration of human IL-9 (100 nM) was injected in all channels orthogonal to the immobilized antibodies. The purified antibodies diluted in running buffer were then injected, and the signal at the start of the dissociation was monitored.

Results and Discussion

The objective was to identify high-affinity antibodies that recognize different epitopes on human IL-9. Twenty supernatants containing mouse anti-human IL-9 antibodies were screened and ranked according to their affinities after their kinetic rate constants were determined. Four antibodies showing high affinities were then purified and their kinetic rate constants reanalyzed. Additionally, epitope mapping was performed to identify antibodies recognizing different epitopes.

Screening and Ranking of Anti-Human IL-9 Supernatants

Twenty supernatants containing anti-human IL-9 antibodies developed in mouse were screened to identify positive clones that bound human IL-9 and to determine the kinetic binding constants of the clones. To capture the antibodies from the supernatant, anti-mouse IgG antibody was first immobilized in six channels. In each cycle, five supernatants were captured and their kinetic constants determined. The captured antibodies were then stripped off the chip to allow capture of five additional supernatant antibodies. Overall, four such cycles were performed in about 1 hr, covering the kinetic analysis of all 20 supernatants.

Of the 20 supernatants, four showed no interaction with human IL-9. Some supernatants exhibited complex kinetic behavior that could not be fit to a simple 1:1 interaction model (Figure 2, clone 9). Isotyping studies revealed that these supernatants contained IgM antibodies, which probably accounted for the deviation from the simple Langmuir interaction (not shown). The kinetic rate constants of all other supernatants were determined, and representative results from five of the antibodies are summarized in Table 1; their fits are shown in Figure 2. The five selected supernatants had similar affinities, though supernatant 2 had a slightly higher affinity (lower equilibrium dissociation constant, K_D) and a relatively lower dissociation rate constant than the others.

Kinetic Analysis of Purified Anti-Human IL-9 Antibodies

After screening and ranking of the 20 supernatants, four anti-human IL-9 antibodies were chosen for further purification: clones 1 and 2 and two subclones of parent clone 7 (termed 7a and 7b). These purified antibodies were then subjected to detailed kinetic analysis. The four antibodies were immobilized on a ProteOn GLC sensor chip using standard amine coupling. Samples of human IL-9 analyte were prepared at six different

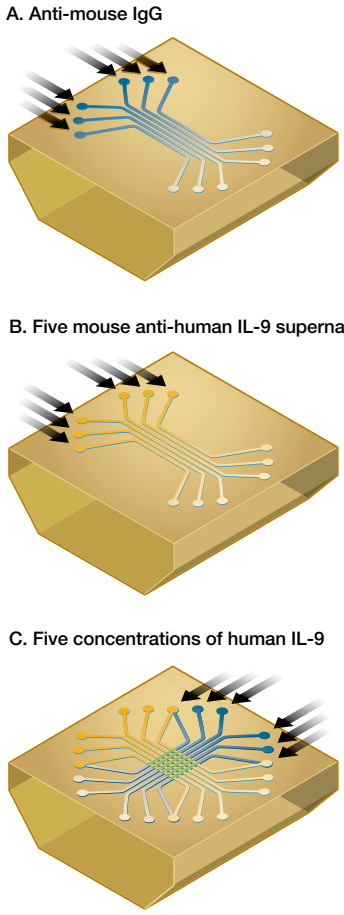


Fig. 1. Reagent flow used for screening and ranking of hybridoma supernatants. A, immobilization of anti-mouse IgG; **B**, capture of five different mouse anti-human IL-9 supernatants (running buffer was injected into the sixth channel); **C**, injection of five concentrations of human IL-9 (running buffer was injected into the sixth channel).

Table 1. Kinetic analysis and ranking* of anti-human IL-9 supernatants of selected clones.

Supernatant	k_a ($M^{-1}sec^{-1}$)	k_d (sec^{-1})	K_D (M)
2	7.0×10^4	4.8×10^{-5}	6.9×10^{-10}
5	8.3×10^4	1.2×10^{-4}	1.4×10^{-9}
3	4.5×10^4	1.1×10^{-4}	2.4×10^{-9}
7	2.1×10^5	6.0×10^{-4}	2.9×10^{-9}
1	6.5×10^4	5.0×10^{-4}	7.7×10^{-9}

* Highest to lowest affinity, in descending order.

concentrations and injected simultaneously over the four antibodies; an empty reference channel was also included. Consequently, a single injection generated six concentration-dependent sensorgrams for each antibody, allowing full kinetic rate constant determination (Figure 3). The data were fitted to a simple 1:1 interaction model, and the kinetic constants thus derived are summarized in Table 2. All four antibodies had equilibrium dissociation constants in the nanomolar range; however, antibodies from clones 1 and 2 had slower dissociation rate constants than subclones 7a and 7b, indicating that the antibody-antigen complexes of antibodies 1 and 2 were more stable.

The kinetic constants derived from the analysis of purified antibodies were comparable to those obtained from supernatants, demonstrating that valuable information can be obtained using unpurified antibodies in supernatants.

Table 2. Kinetic analysis of purified anti-human IL-9 antibodies.

Clone	k_a ($M^{-1}sec^{-1}$)	k_d (sec^{-1})	K_D (M)
1	5.3×10^4	9.2×10^{-5}	1.7×10^{-9}
2	7.1×10^4	6.3×10^{-5}	8.9×10^{-10}
7a	2.0×10^5	7.9×10^{-4}	4.0×10^{-9}
7b	1.9×10^5	5.6×10^{-4}	2.9×10^{-9}

Epitope Mapping of Purified Anti-Human IL-9 Antibodies

In the next step, epitope mapping of the four purified antibodies was performed to determine if these antibodies recognized different or similar epitopes. For this purpose, the same ProteOn GLC chip containing the four immobilized antibodies was used. A single concentration of human IL-9 was injected in the analyte orientation (over the four immobilized antibodies), resulting in formation of antibody-antigen complexes. This step was followed by an immediate injection of the four antibodies (also in the analyte orientation), forming a sandwich complex of antibody-antigen-antibody. The level of binding of the second set of antibodies was monitored at the start of the dissociation phase, and the values obtained are summarized in Table 4. All values were normalized according to the signals obtained when the same antibody was used both as the first and second antibody.

The results indicate that antibodies 1 and 2 shared the same or overlapping epitopes, since no binding was observed when these two were tested pairwise. The same was true for antibodies 7a and 7b; however, the epitope recognized by antibodies 1 and 2 was different from that recognized by 7a and 7b (Table 3). Therefore, these two epitopes were located at distinct places on the human IL-9 antigen (Figure 4).

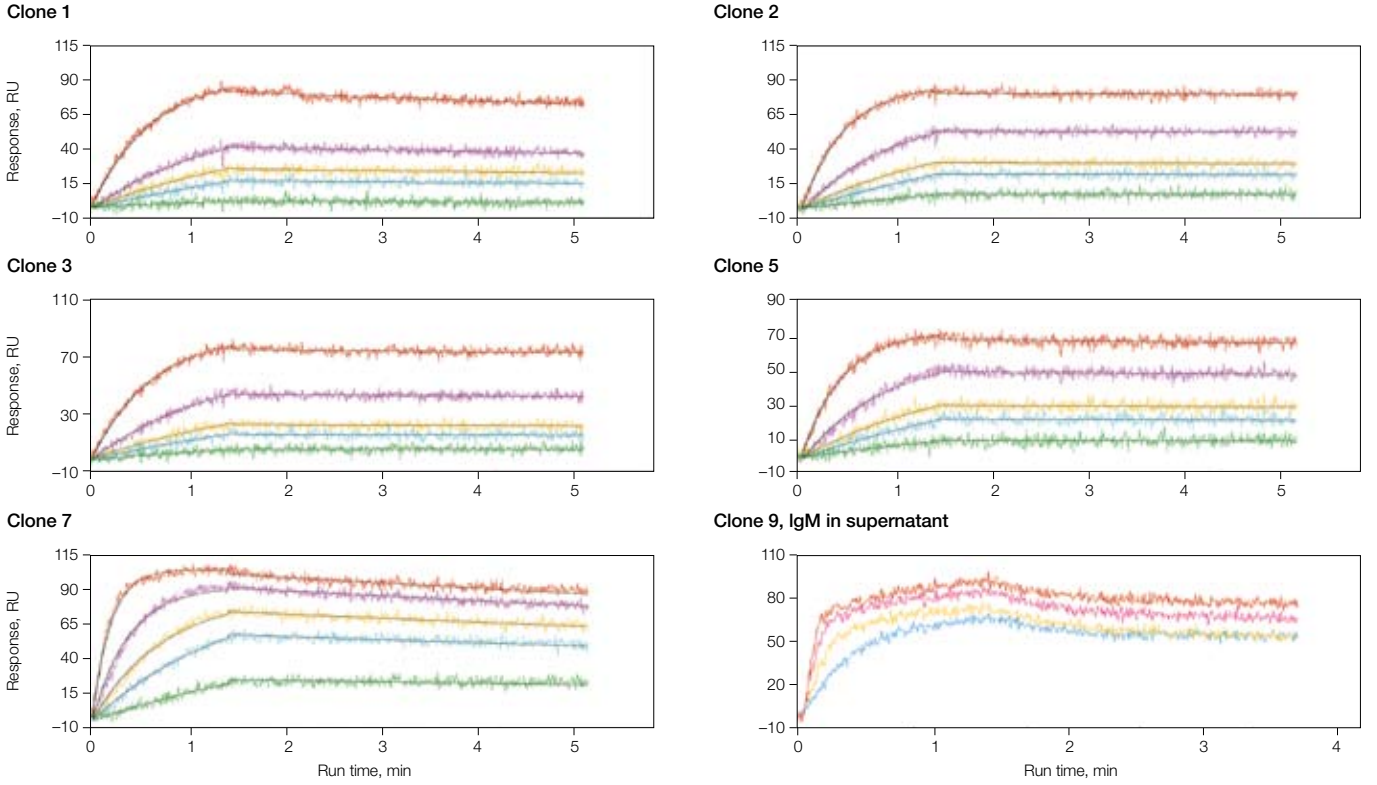


Fig. 2. Screening of mouse-anti-human IL-9 supernatants. Shown are sensorgrams generated by the interactions between six different anti-human IL-9 supernatants with five concentrations (—, 100 nM; —, 50 nM; —, 25 nM; —, 12.5 nM; —, 6.25 nM) of human IL-9. Black lines represent the global fit of the sensorgrams to a 1:1 interaction model. See Table 1 for the kinetic rate constants derived from these data.

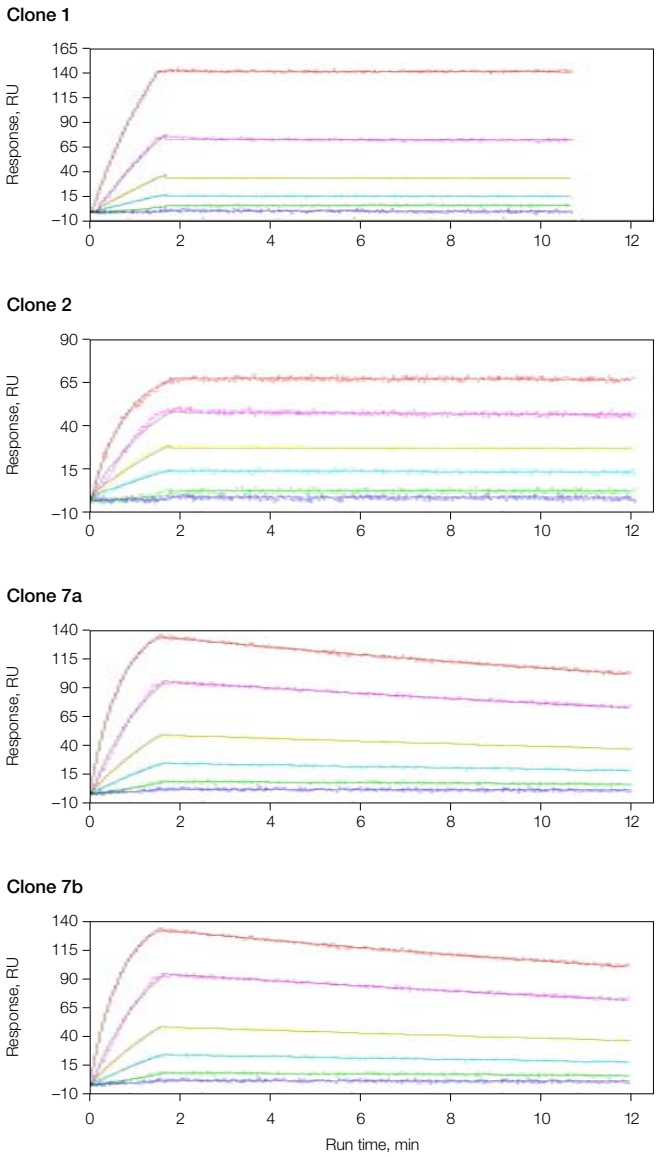


Fig. 3. Kinetic analysis of the IL-9 antibody/IL-9 interaction. Shown are sensorgrams generated by the interaction of four purified anti-human IL-9 antibodies with six concentrations of human IL-9 (—, 100 nM; —, 50 nM; —, 25 nM; —, 12.5 nM; —, 6.25 nM; —, 3.18 nM). Black lines represent the global fit of the sensorgrams to a 1:1 interaction model. See Table 2 for the kinetic rate constants derived from these data.

Conclusions

This study demonstrates the rapid, precise screening and ranking of 20 hybridoma supernatants using the ProteOn XPR36 system. Five high-affinity antibodies were identified, and four of these were selected for further purification and epitope mapping. Two of the antibodies recognized different epitopes on the IL-9 antigen. Both epitope mapping and hybridoma ranking were accomplished efficiently using the parallel processing capability of the ProteOn XPR36 system. This work can be easily enhanced to include kinetic constant determination of hundreds of supernatants within only a few hours.

In the workflow used in this study, five supernatants were screened at a time against five antigen concentrations, and one channel was reserved for referencing. Using this configuration, both the anti-mouse IgG and antibody-containing supernatants were injected into the same set of channels (ligand channels), followed by IL-9 injection into the orthogonal analyte channels (Figure 1). New applications being tested, however, have shown that by using a different configuration for sample flow and interspot referencing, all six available analyte channels may be used for supernatant screening, yielding equally reliable results with greater throughput. This is accomplished by using the ligand channel for immobilization of anti-mouse IgG, the analyte channel for capture of the supernatant clones, and the ligand channels for IL-9 flow. For more information, refer to the ProteOn XPR36 Application Guide, bulletin 5412.

For additional copies of this article, request bulletin 5540.

Table 3. Epitope mapping of purified anti-human IL-9 antibodies. Values represent signals observed at the start of the dissociation phase after normalizing against signals observed when the same antibody was used as both the first and the second antibody. Colors represent antibody pairs (interacting once as the first antibody and once as the second antibody).

Second Antibody \ First Antibody	2	1	7a	7b
2	1	**0.2**	5	6
1	**0.3**	1	3	4
7a	49	27	1	**1**
7b	44	19	**1**	1

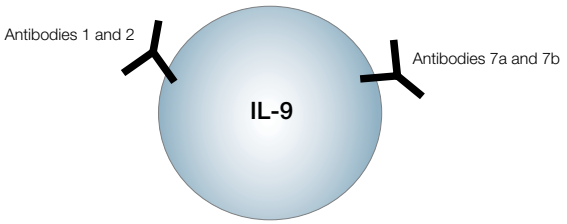
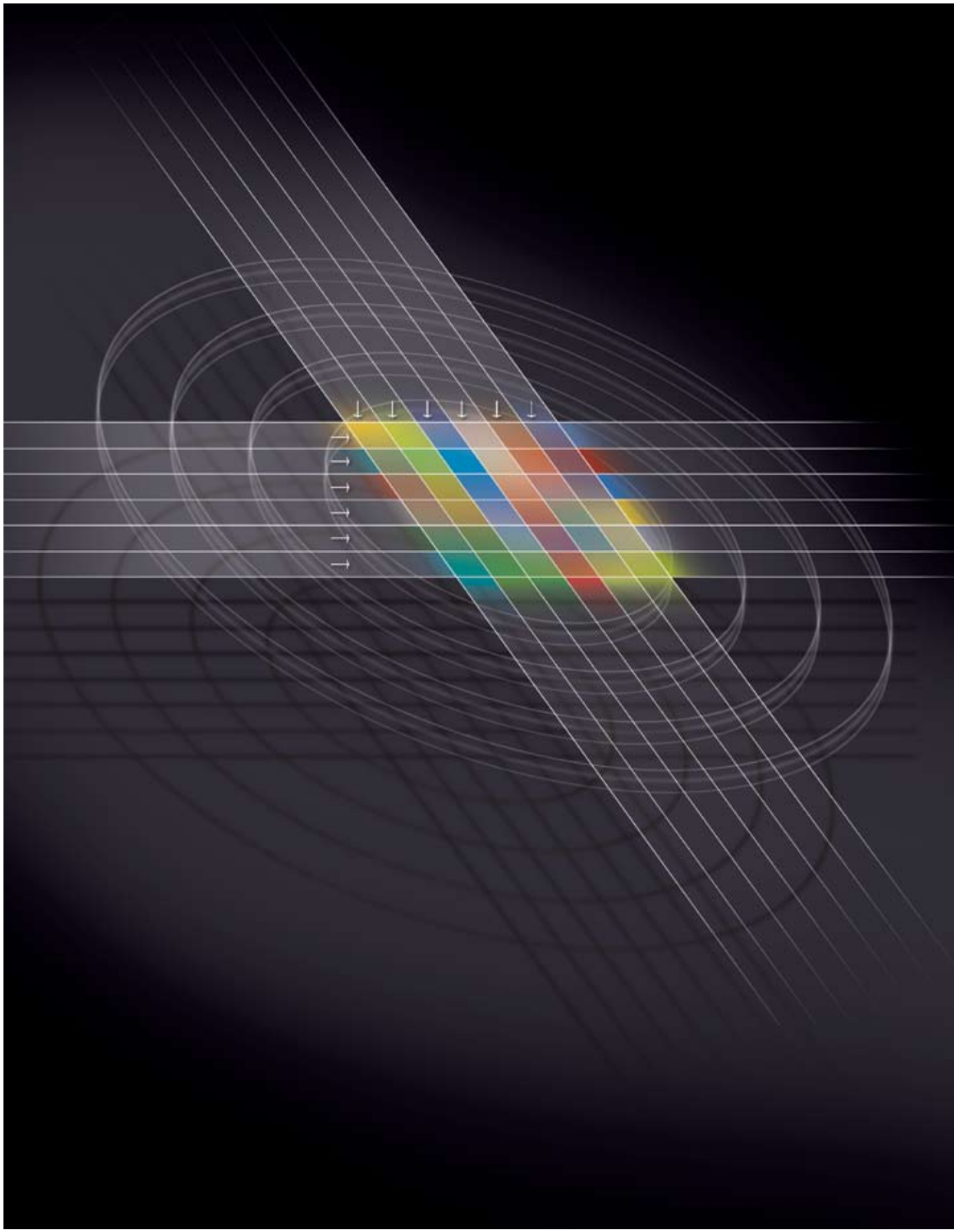


Fig. 4. Representation of the results of epitope mapping. Antibodies 1 and 2 share the same or overlapping epitopes, as do antibodies 7a and 7b. However, the epitope recognized by antibodies 1 and 2 is different from that recognized by 7a and 7b.



Authors: Teresa Esch, Jill Raymond, Ariel Notcovich, Gideon Schreiber, Laëtitia Rubrecht, Steve Lipson, and Emily Dale

Accelerating Research and Discovery With the ProteOn™ XPR36 Protein Interaction Array System

As proteomics research gains momentum and the interest in using proteins as therapeutics and in mutational studies grows, surface plasmon resonance (SPR) technology is becoming increasingly prominent. This technology enables researchers to study the kinetics of protein interactions by measuring small changes in refractive index that are induced by interaction partners at the surface of the chip. The rapid advancement of this technology is enabling scientists to address and answer more complex questions in less time.

The ProteOn XPR36 protein interaction system is at the forefront of this development. This SPR optical biosensor includes a unique crisscross microfluidics design, which allows parallel processing of samples and enables a new application termed “one-shot kinetics”. Using the ProteOn XPR36 system, scientists can acquire kinetic, specificity, and affinity data from 36 biomolecular interactions in one rapid experiment. Because multiple conditions can be tested simultaneously, the process of optimizing protocols for immobilization and binding reactions can be handled quickly and efficiently, without the need to regenerate the reaction surface between samples. This means less time spent optimizing conditions, and more time spent running experiments and analyzing data.

For Gideon Schreiber and Laëtitia Rubrecht, two scientists who use the ProteOn XPR36 system, the new applications made possible by the instrument’s parallel approach to SPR translate into increased efficiency, flexibility, and opportunities for scientific breakthroughs. For Ariel Notcovich, one of the creators of the ProteOn system, such stories are the rewards of 6 years of hard work.

Gideon Schreiber Protein Interactions and the Nature of Protein-Protein Interfaces

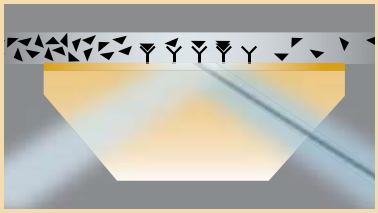
Gideon Schreiber, a professor at the Weizmann Institute in Israel, was one of the first scientists to use the parallel processing capability of the ProteOn XPR36 instrument. A biochemist, Schreiber became interested in studying protein-protein interactions because, he says, these are "what is driving life". He examines protein binding interfaces to determine what roles different amino acids play in proteins' interactions, and how small differences in a specific set of amino acids forming a binding interface might lead to the differential effects of various ligands acting through the same receptor. This information could be used to alter interfaces to change affinity or specificity, thereby modifying the effect of protein interactions.

To study protein interfaces, Schreiber mutates specific amino acids in binding sites and uses SPR analysis to measure the resulting changes in the binding kinetics. He creates mutations in the ligand, the receptor, or both, and examines how these changes affect interactions under various binding conditions. A thorough investigation of the binding interface between two proteins can involve thousands of different combinations of mutations and conditions. Schreiber says measuring the binding kinetics under all the possible combinations was impossible without the parallel processing capability of the ProteOn instrument. Now Schreiber can rapidly collect all the data he needs to understand the components of binding sites.

Schreiber is a strong proponent of data-driven hypotheses. He compares data collection to shining a flashlight on a piece of paper: The bigger the light, the more data; the more data, the better you will be able to decipher what is there. The ProteOn system has allowed him to generate large amounts of data and has already led him to a novel hypothesis about how protein interfaces are constructed. He has found that these interfaces are made up of a number of discrete modules, each of which comprises a subset of the amino acids of the interface. There is little interaction between the modules, so any of the amino acids in one module can be mutated without affecting interactions at other modules. Furthermore, different ligands appear to interact with a receptor via different, but overlapping, sets of modules. According to Schreiber, this model of protein interfaces would not have come about without the large amount of data he has been able to collect using the ProteOn system.

In addition to appreciating the high throughput of the ProteOn system, Schreiber says the "data is reliable and easy to work with". He adds that anyone can learn to use the instrument in a day. Schreiber points out, however, that he has been working closely with the instrument's development team starting with some of the earliest prototypes. "So I may be biased", he says, and smilingly adds, "although I am not".

SPR and One-Shot Kinetics With the ProteOn System



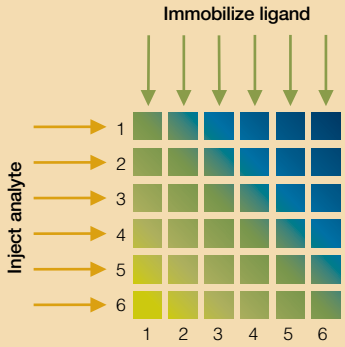
The ProteOn XPR36 system allows researchers to run up to 36 protein kinetics experiments in one shot — a major improvement that the system's developers have termed "one-shot kinetics". Here's how SPR and one-shot kinetics work.

SPR occurs when light interacts with a metal film placed at the interface between two materials that have different refractive indices (such as glass and buffer). When light strikes the glass at a specific angle, called the SPR angle, some energy is transferred to the metal surface. This absorbed energy generates a wave of electrons (plasmons) along the metal surface, which causes a decrease in the intensity of reflected light. An optical sensor can measure this decrease, termed the SPR dip, even when it is very small (corresponding to a change in refractive index of 1 x 10^{-6}).

For proteomic research, measuring the SPR dip in a buffer solution provides accurate concentration, thermodynamic, and binding kinetics data about protein-protein interactions. A buffer/glass interface separated by gold film, called a sensor chip, is coated with a reactive surface, and serial dilutions of a ligand are allowed to bind to it. Serial dilutions of a second protein (analyte) are then injected into the buffer. Interaction between the analyte and the ligand changes the refractive index at the interface sufficiently to create a measurable SPR dip.

Traditional analysis techniques use SPR serially: one interaction followed by another. Now, using one-shot kinetics with the ProteOn XPR36 system, researchers can rapidly collect interaction data on two proteins at up to six concentrations or up to 36 interactions measured in one experiment. A key advantage of one-shot kinetics is its accurate, precise, and reproducible determination of binding kinetics. Sensor chips may be regenerated after each 6 x 6 experiment for further analysis. With one-shot kinetics, researchers can reliably analyze protein-protein interactions without the trouble of duplicating conditions or regenerating fragile ligands between experiments.

Refer to the feature article in *BioRadiations 119* for more information on use of SPR and the ProteOn XPR36 system for analysis of protein interactions.



Simple setup of one-shot kinetics. Step 1, bind different concentrations of ligand in each channel of the sensor chip. Step 2, inject various concentrations of analyte into orthogonal channels. Step 3, analyze SPR data using interspot regions as local references.

Laëtitia Rubrecht Characterization of Antibodies for Biomarker Assays

Laëtitia Rubrecht works at the Complex System Modeling and Engineering for Diagnostics Laboratory in Montpellier, France. She works in a laboratory that develops diagnostic assays using antibodies against early markers for diabetes, heart disease, and neurological disorders.

Rubrecht's laboratory develops antibody pairs to screen disease biomarkers in Bio-Plex® assays. Bio-Plex assays are sandwich assays, requiring two antibodies that bind to different epitopes of the same target molecule. To create a multiplex assay, therefore, one must first identify two complementary antibodies against each biomarker to be assayed (see sidebar).

…the parallel processing capability of the ProteOn instrument allows Rubrecht to directly compare multiple antibodies under identical conditions…

The antibody selection process is a daunting task, involving multiple repeated trials and some guesswork. First, thousands of supernatants containing monoclonal antibodies are generated. Next, ELISAs are used to narrow the selection to approximately 50 candidates. From these 50 candidates, 10 are chosen for purification and further characterization. That's where the guesswork comes in — there is no systematic method to ensure that the 10 antibodies selected for characterization are the best available for getting antibody pairs. This is because sandwich ELISAs cannot be performed using unpurified antibodies.

The ProteOn system provides Rubrecht with a more rational approach to antibody selection, giving her more confidence in her choice of the 10 best. She now bases her selection on the affinity of the antibodies — information that the ProteOn instrument provides but ELISAs do not. Furthermore, the parallel processing capability of the ProteOn instrument allows Rubrecht to directly compare multiple antibodies under identical conditions, without the possibility of artifacts from slight variations in buffer or from ligand degradation during chip regeneration. Finally, Rubrecht highlights the fact that in using the ProteOn system, supernatants can be tested without initial purification steps, a capability she emphasizes as "very important". All these features combine to make the antibody selection process faster, easier, and more robust.

Using the ProteOn System to Accelerate the Development of Bio-Plex Assays

For more than 50 years, Bio-Rad has been introducing new tools for protein analysis — tools that can work together to provide solutions for nearly every step of your proteomics workflow. One such workflow solution is Laëtitia Rubrecht's use of the ProteOn XPR36 protein interaction array system to screen antibodies when developing multiplex immunoassays for the Bio-Plex 200 multiplex suspension array system.

The Bio-Plex system is a flexible, easy-to-use suspension array system that permits simultaneous analysis of up to 100 different disease biomarkers in a single microplate well. Bio-Plex assays use sets of spectrally coded, internally dyed beads conjugated with antibodies to detect specific target proteins or peptides. The antibody-conjugated beads are allowed to react with a sample, and then a fluorescently labeled detection antibody is added to form a capture sandwich immunoassay, with the binding pattern bead–antibody–peptide–secondary antibody–fluorescent label. Bio-Plex multiplex assays are created by mixing sets of beads conjugated with different antibodies to simultaneously quantify many analytes in one sample.

A key challenge in developing any multiplex immunoassay is finding high-affinity antibody pairs that are also very specific. This eliminates cross-reactivity among the various antibodies. Before Rubrecht had a ProteOn instrument in the laboratory, she worked with purified samples, which was a time-consuming process. Additionally, since the purification process could not be performed on every candidate, Rubrecht would choose the 10 best candidates from the limited information available. The addition of the ProteOn instrument to the laboratory saves time by allowing crude samples to be run. It also provides more complete information, allowing Rubrecht to make a more rational choice of the 10 best candidates.

The ProteOn and Bio-Plex systems in the laboratory:

Step 1: Narrow supernatant clones to those that appear to bind well to the target using ELISA assays.

Step 2: Perform secondary screening for affinity and possible complementarity using the ProteOn XPR36 system.

Step 3: Purify and label selected clones, then test using ELISA sandwich assays.

Step 4: Validate the best candidates using the Bio-Plex system.

Step 5: Screen normal and disease samples using Bio-Plex assays in the Bio-Plex system.

ProteOn XPR36 system

Bio-Plex 200 system

Sample testing

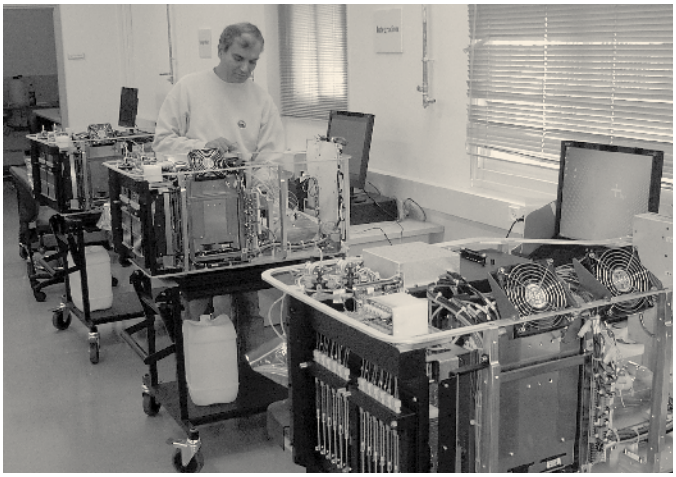
Ariel Notcovich Development of the ProteOn System

As a graduate student in the laboratory of Professor Stephen Lipson at the Technion-Israel Institute of Technology, Ariel Notcovich learned the usefulness of using refractive index measurements to study complex systems. For his doctoral thesis, he hoped to use interferometric microscopy to measure electrical activity and the establishment of connections in cultured neural networks. But he quickly found that the interferometric microscope he was using was not sensitive enough to measure the subtle changes in refractive index that occurred in cells. Undeterred, Notcovich turned to SPR technology, a "simple nonlinear optical effect" that greatly amplifies small changes in refractive index, to boost the signal.

When Notcovich, with his experience in microscopy, was introduced to the power of SPR, he had a novel idea: "Why don't we build an interference microscope that uses SPR?"

With the help of a technician, and using old microscope parts, Notcovich built his first SPR phase imaging system — an instrument capable of making SPR measurements from an image. This led to patent approval through Professor Lipson and Technion-Israel Institute of Technology. Thus began a 6 year detour from his doctoral research that culminated in the creation of what became Bio-Rad's ProteOn XPR36 protein interaction system.

Notcovich quickly realized that his SPR microscope would be useful to many types of researchers. Protein researchers, for example, had already been using SPR biosensors to measure protein binding kinetics, but were only able to measure one reaction at a time. The design developed by Notcovich and his colleagues — particularly the ability to make SPR measurements on images — permitted measurement of hundreds of interactions at once, potentially changing the experimental design of SPR kinetic analysis.



Working with collaborators from many fields — biologists, physicists, chemists, chemical engineers, and software engineers — Notcovich continued developing and improving prototypes until the design for the ProteOn instrument was developed. Experts from other fields contributed additional innovations, such as the multichannel module, which creates the 6 x 6 interaction array pattern and makes it possible to analyze up to six ligands and six analytes simultaneously and in parallel.

> **"Why don't we build an interference microscope that uses SPR?"**
>
> **—Ariel Notcovich**

Previous SPR methods required regeneration of the chip after each reaction, which can damage the ligand or introduce other variations that must be taken into account during data analysis. Because the ProteOn instrument allows 36 interactions to be performed on the same chip at the same time under the same conditions, researchers can have more confidence in the results. Local referencing using interspot regions further increases confidence, because any kind of local effect can be subtracted or corrected. Investigators can develop hypotheses and conduct further research with fewer false starts and dead-ends.

When Notcovich explains the one-shot kinetics approach to people who are familiar with using SPR, they "immediately can appreciate how their lives will be enriched" by using this multiplexing approach. For other people, it "takes a little time, but suddenly — you can see a smiley face, and they say, 'This is great. I can do that and I can do this and I can see concentrations and I can do that in one shot.' And you don't have to worry about your experimental conditions — you just do it."

Bringing an Idea From Academia to Market

Stephen Lipson, a professor of physics at Technion-Israel Institute of Technology and Ariel Notcovich's mentor, is well positioned to reflect on differences between academia and industry. Of the 30 or so graduate students he has mentored in the last 40 years, about 80% have gone on to careers in industry. Before Notcovich invented his SPR microscope, two other technologies developed in Lipson's laboratory spawned new companies — CI Systems, a company that makes infrared-measuring instruments for the semiconductor industry, and Applied Spectral Imaging (ASI), which makes instrumentation used for mapping chromosomes. After he and Notcovich wrote their patent, Lipson was excited to have the opportunity to help his student start a new company.

Setting up the new company was an adventure for Lipson, but it was also a bit frustrating at times. In the initial stages, he and Notcovich had to convince venture capitalists to invest in a new, unproven technology. The investors were necessarily concerned with the profitability of their investment, but Lipson was so excited by the ideas behind the instrument, he had difficulty focusing on these business considerations. Equally excited by the opportunity in SPR, Bio-Rad partnered with Notcovich and Lipson to codevelop the current product.

An even bigger frustration, however, was having to modify the original optical design after several months of work. "We were emotionally connected with that idea because this, we thought, was the most wonderful idea. And it turned out it wasn't as wonderful as we thought and we had to change our frame of mind about it." This is a common problem when you are developing a product, Lipson believes. "There are ideas that you have that turn out not to be the best way of doing things and you have to … change your mind. And that's what we did and it turned out to be very profitable."

Still, Lipson prefers working in academia. He enjoys taking an idea and developing it into something useful, but in industry you have to focus on what makes an instrument viable; you have to make it valuable to other people. "I like to make it interesting for me and not worry about other people. But in industry you can't do that", he says.

For Notcovich, starting a new company meant taking a 5-year hiatus from his PhD work. Nonetheless, he feels it "was a great experience". He is now returning to his graduate studies of neural networks, aiming to receive his doctorate in the next 2 years. After that, he would like to continue working in a multidisciplinary environment to develop new instruments for the life sciences. He believes that the extensive collaboration among scientists from various disciplines is one of the great advantages of working in industry.

Notcovich encourages other academics to bring their good ideas to market. For those who want to travel this path, he advises, "Think of the right ideas and push your ideas. But get the help of other people and link yourself to strategic partners that will help you move from the lab to market — it is a long way."

Notcovich considers bringing a concept designed from spare parts in a laboratory "from zero to commercialization" his greatest accomplishment. He also appreciates the role that Bio-Rad has played in helping him bring the product to commercialization by funding the development over the last several years. He looks back over the labors of the last 6 years, and "suddenly it looks like 6 months and not 6 years". He reflects on how this project that he started as a PhD student "working day and night" by himself in the laboratory grew into a project involving many scientists, engineers, and other specialists. Now there are people around the world "supporting this instrument, teaching about it, and training other people, and of course, it's much bigger than the original idea. Many people have good ideas to commercialize, but without a strategic partner like Bio-Rad, many of those ideas would stay in a drawer."

Conclusions

Bio-Rad's ProteOn XPR36 protein interaction array system started as the idea of a doctoral student in optical physics. Seeing the potential to help other scientists, Ariel Notcovich and his mentor, Stephen Lipson, began a multidisciplinary collaboration to create the final product. Bio-Rad's later involvement accelerated the development of the ProteOn instrument and led to the commercialization of the final product.

Unlike previous SPR-based technology that limited experiments to single reactions at a time, the ProteOn system's parallel processing enables 36 protein interactions to be measured simultaneously. This novel approach has greatly increased the amount of data that a researcher can output, has improved the quality of data, and has already greatly impacted the research of protein scientists.

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Applications of the ProteOn™ NLC Sensor Chip: Antibody-Antigen, DNA-Protein, and Protein-Protein Interactions

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Introduction

The ProteOn NLC sensor chip is one of several types of sensor chips designed for use with the ProteOn XPR36 protein interaction array system. The chip is coated with a layer of immobilized NeutrAvidin used for single-step immobilization of various biotinylated biomolecules (for example, peptides, proteins, oligonucleotides, and carbohydrates). The neutral charge of this NeutrAvidin layer minimizes nonspecific binding, and also enhances immobilization of oligonucleotides because their negative charge does not interfere with surface immobilization. Terminal labeling of immobilized oligonucleotides with biotin ensures high accessibility to analyte molecules, and no surface activation or deactivation steps are required, making the NLC immobilization protocol both rapid and effective.

In this technical report we demonstrate the use of the ProteOn NLC sensor chip and surface plasmon resonance (SPR) with the ProteOn XPR36 system (Figure 1) to determine the kinetic rate constants for three bimolecular model interactions: 1) cytokine IL-2 with anti-IL-2 antibody (antigen-antibody interaction), 2) *Escherichia coli* tryptophan (*trp*) operator sequence with *trp* repressor protein (DNA-protein interaction), and 3) interferon-α2 (IFNA2) with interferon receptor IFNAR2 (protein-protein interaction). For each model interaction, six concentrations of analyte were injected across five channels of immobilized biotin-labeled ligand in a single automated step, generating six sets of five analysis sensorgrams. These sensorgrams were fitted with a global 1:1 interaction model to yield kinetic constants using the one-shot kinetics technique (Bronner et al. 2006).

Methods

Instrumentation and Reagents

The ProteOn XPR36 protein interaction array system was used for all experiments, and each of the three model interactions was studied on a separate NLC sensor chip. Biotinylated ligand was immobilized at a constant concentration in five of the six ligand channels; the sixth channel was used as a reference channel. ProteOn phosphate buffered saline, pH 7.4 with 0.005% Tween 20 (PBS/Tween) was used as diluent and running buffer. Experiments were performed at 25°C.

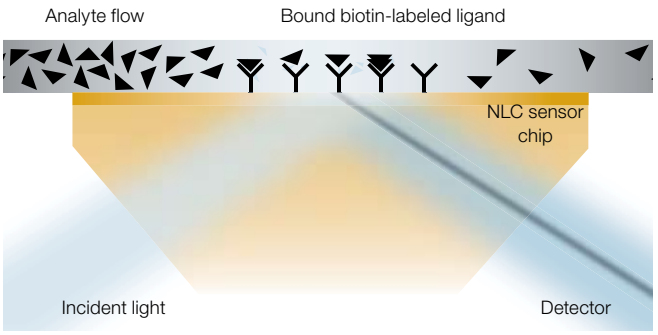


Fig. 1. Surface plasmon resonance and the ProteOn NLC sensor chip. Analyte molecules bind to biotin-labeled ligands at the surface of the ProteOn NLC sensor chip, causing a shift in the SPR response curve. The shift is proportional to the mass change near the NLC sensor chip surface.

Antigen-Antibody Interaction

Ligand — Mouse anti-human IL-2 antibody (BD Pharmingen) was biotinylated with biotin-NHS ester (Sigma) and immobilized in five channels of a ProteOn NLC sensor chip at 25 µg/ml (167 nM) in PBS, pH 7.4 at a flow rate of 30 µl/min. The sixth channel was used as a reference channel.

Analyte — Analyte samples of human cytokine IL-2 (BD Pharmingen) were prepared at concentrations of 80, 40, 20, 10, 5, and 2.5 nM by serial dilution in PBS. Following initial preconditioning of the sensor chip surface with a short injection of 0.85% phosphoric acid solution, the six IL-2 samples were injected into the six analyte channels at a flow rate of 100 µl/min. The injection step included a 1 min association phase followed by an 11 min 40 sec dissociation phase in PBS, which was used as the continuous-flow buffer throughout the experiment.

DNA-Protein Interaction

Ligand — A biotinylated, single-stranded 3-mer oligonucleotide containing the *trp* operator sequence (5'-biotin-TTTTTTTTTAT-GCTATCGTACTCTTTAGCGAGTACAACCGGGG, Sigma) was immobilized in five channels of a ProteOn NLC sensor chip at a concentration of 0.5 µM in PBS, pH 7.4 at a flow rate of 30 µl/min. The unlabeled complementary strand (0.5 µM) was injected into the same channel, and hybridization was monitored (~35% hybridization was achieved). Running buffer was injected into the sixth channel, which was used as a reference channel.

Analyte — Samples of *trp* repressor protein (Panvera) were prepared at concentrations of 8, 6, 4, 2, 1, and 0.5 nM by dilution in PBS/Tween containing 4 mM tryptophan. Samples of each concentration were injected into the six analyte channels at a flow rate of 40 µl/min. The injection step included a 1 min 30 sec association phase followed by a 3 min 20 sec dissociation phase in PBS with 4 mM tryptophan, which was used as the continuous-flow buffer throughout the analyte interaction step.

Protein-Protein Interaction

Ligand — The interferon receptor IFNAR2-EC (25 kD) (provided by G Schreiber, Weizmann Institute) was labeled with *N*-biotinyl-6-aminocaproic acid-*N*-succinimidyl ester (Fluka). The biotinylated IFNAR2-EC (0.2 µM) was immobilized on an NLC sensor chip in five ligand channels in PBS, pH 7.4 at a flow rate of 30 µl/min. The sixth channel was used as a reference channel.

Analyte — Samples of IFNA2 (provided by G Schreiber) were prepared at concentrations of 100, 50, 25, 12.5, 6.25, and 3.12 nM by serial dilution in PBS. Samples of each concentration were injected into the six analyte channels at a flow rate of 30 µl/min. The injection step included a 4 min association phase followed by a 5 min dissociation phase in PBS, which was used as the continuous-flow buffer throughout the experiment.

Sensorgram Acquisition and Data Analysis

Each of the three model interactions was studied on a separate ProteOn NLC sensor chip. The interactions of the six analyte concentrations with one concentration of immobilized ligand were monitored in parallel, generating six sets of five analysis sensorgrams. Two different types of referencing were used for each of the model interactions: interspot referencing (measurement of two inactive spots adjacent to each of the 36 interaction spots) and a reference channel (measurement along the sixth channel, without bound ligand) (Figure 2). Sensorgram correction and kinetic model fitting were applied separately for each of these two types of referencing. Each set of six reference-subtracted sensorgrams was fitted globally to curves describing a homogeneous 1:1 bimolecular reaction model. Global kinetic rate constants (k_a and k_d) were derived from this reaction model.

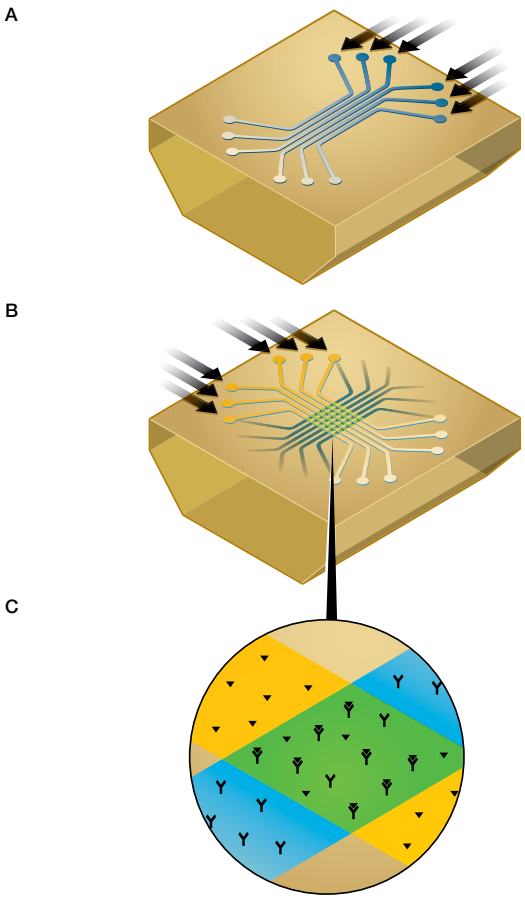


Fig. 2. The ProteOn NLC sensor chip's 6 x 6 interaction array. A, biotinylated ligand is immobilized in five parallel channels, with the sixth channel used as a reference channel; **B**, six different concentrations of analyte are injected into the six orthogonal channels, generating the 36-element interaction array; **C**, detail of a single ligand-analyte interaction spot (green) showing the positions of the two interspot references (yellow).

Results and Discussion

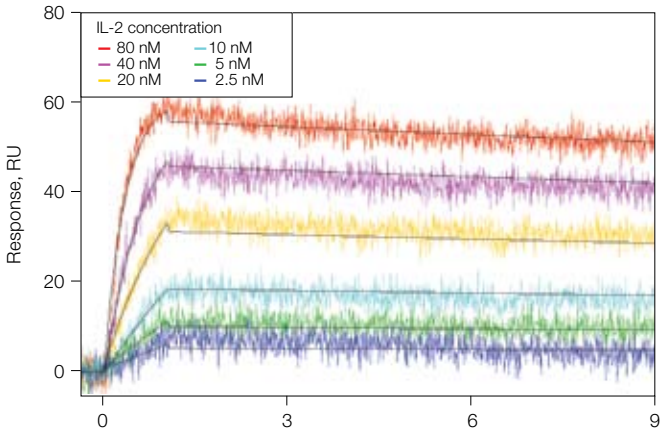
Uniformity of Ligand Immobilization

The immobilization level (ligand density) of each biotinylated ligand was uniform at the six protein interaction spots in each of the six ligand channels (CV ≤3%) (Table 1). These results indicate that each protein interaction spot within each ligand channel acts as an equivalent immobilization surface, ensuring accurate measurement of ligand density and interaction kinetics.

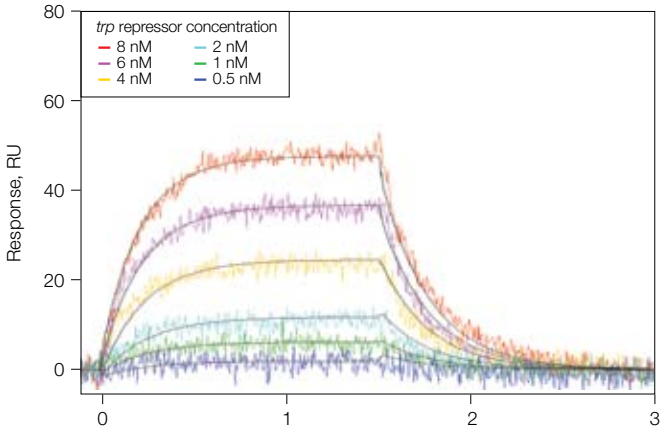
Table 1. Summary of kinetic constants derived for the three model interactions. The equilibrium dissociation constant, K_D, was calculated from k_d/k_a.

		Interspot Reference Subtraction			Reference Channel Subtraction		
Model	**Ligand Density (RU)**	**k_a ($M^{-1}sec^{-1}$)**	**k_d (sec^{-1})**	**K_D (M)**	**k_a ($M^{-1}sec^{-1}$)**	**k_d (sec^{-1})**	**K_D (M)**
IL-2	1,685 ± 1.8%	5.1×10^5	1.1×10^{-4}	2.2×10^{-10}	6.6×10^5	1.9×10^{-4}	2.9×10^{-10}
trp repressor	293 ± 3.0%	1.4×10^6	5.8×10^{-2}	4.1×10^{-8}	1.5×10^6	6.6×10^{-2}	4.4×10^{-8}
IFNAR2	234 ± 2.7%	2.7×10^6	1.4×10^{-2}	5.2×10^{-9}	2.3×10^6	1.2×10^{-2}	5.2×10^{-9}

A. IL-2 and IL-2 antibody



B. *trp* repressor and *trp* operator



C. Interferon-α2 (IFNA2) and interferon receptor IFNAR2

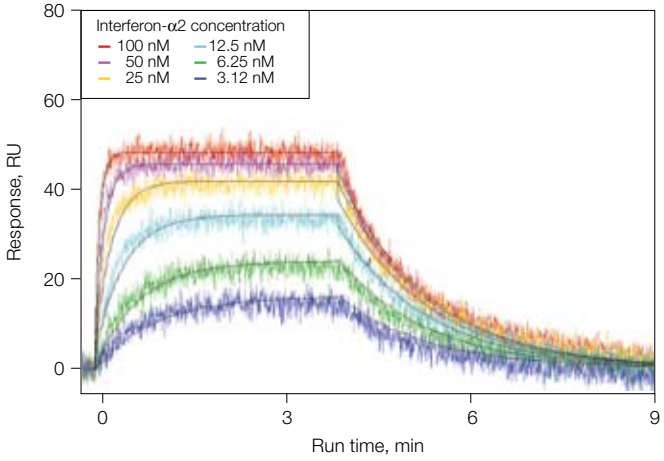


Fig. 3. Three sets of sensorgrams showing three model interactions. Each set of six sensorgrams corresponds to the responses of six analyte concentrations interacting with immobilized ligand. **A**, cytokine IL-2 interaction with anti-IL-2 antibody; **B**, *trp* repressor protein interaction with the *trp* operator sequence; **C**, interferon-α2 (IFNA2) with the interferon receptor IFNAR2. The black lines depict global 1:1 interaction curve-fitting models for each of the three interactions.

Determination of Kinetic Rate Constants

Table 1 summarizes the results for kinetic constants of association (k_a) and dissociation (k_d) derived from each of the three model interactions. Figure 3 shows representative sensorgrams fitted to 1:1 global curves for each model interaction. The residual error for each fit was <10% of the associated R_{max} value (data not shown).

Reference Subtraction

As described in Methods, two different types of reference sensorgrams were used for each interaction. Sensorgram correction and kinetic model fitting were performed separately for each reference type. Kinetic constants derived from each referencing method were similar (Table 1), demonstrating the equivalence of the two reference models and validating the use of interspot references as a rigorous empirical tool. The use of interspot references is especially useful in experimental protocols where high throughput and greater experimental flexibility are desired (Bronner et al. 2006).

Conclusions

In this technical report we demonstrate the determination of kinetic rate constants for three typical bimolecular model interactions using one-shot kinetics with the ProteOn NLC sensor chip. Each of the three models studied used a simple two-step protocol: immobilization of one concentration of biotinylated ligand, followed by injection of six different concentrations of analyte. The results demonstrate the efficiency of the one-shot kinetics technique described in detail by Bronner et al. (2006), and because interspot references eliminate the need for using a reference channel, throughput and flexibility are maximized for those experimental designs in which multiple ligands are under investigation.

Acknowledgements

We thank Gideon Schreiber from the Department of Biological Chemistry, Weizmann Institute, Rehovot, and Tali Haran from the Biology Department, Technion, Haifa, for their collaboration in this study.

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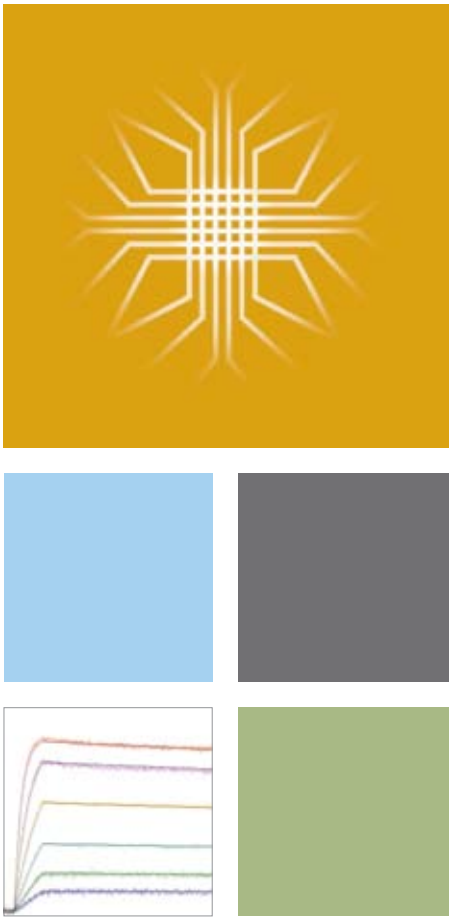
For additional copies of this article, request bulletin 5449.

Protein Interaction Analysis



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Using the BioOdyssey™ Calligrapher™ MiniArrayer to Form Immobilized Protein Microarrays on Surface-Modified Glass Substrates

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Introduction

The development of adequate material platforms for high-throughput assays has become an important issue in the field of biotechnology, because such platforms can accelerate genomic, proteomic, and disease-diagnosis studies (Drickamer and Taylor 2002, Huang 2001). Furthermore, these platforms have significant potential to contribute to the advancement of drug discovery, tissue engineering, and stem cell technologies. Substrate materials that are commonly used in this area of research are glass, polystyrene, stainless steel, polypropylene, and gold. In order to incorporate suitable surface chemistries onto these substrates, various coating processes have been adopted, including dip or spin coating, surface grafting, plasma polymerization, chemical vapor deposition, self-assembled monolayers, and layer-by-layer deposition methods. Such functional substrate surfaces then allow the immobilization and study of specific individual biological factors — particularly those factors that can control the behavior of mammalian cells — in a microarray format (Anderson et al. 2004). The immobilization of combinations of such factors (i.e., cocktails) is also possible and enables true combinatorial studies.

We have designed cell microarray substrate coatings that provide a low cell-attachment background, functional groups for the covalent immobilization of biologically active signals, and excellent adhesion to the microarray substrate material (Kurkuri et al. in press, Thissen et al. in press). We used coatings made of random copolymers of glycidyl methacrylate (GMA) and polyethylene glycol methacrylate (PEGMA), which were deposited onto glass slides that had been previously coated with an allylamine plasma polymer (ALAPP) as a pinning layer. In the copolymer we used, GMA provided reactive oxirane groups for both biomolecule immobilization and attachment to the underlying ALAPP layer, while the other component, PEGMA, was thought to provide a low cell-attachment background. Different methods of attaching the copolymer to the underlying ALAPP layer (dip coating, spin coating, and grafting) were compared using surface analytical techniques.

In this study, a model protein (fluorescein isothiocyanate-labeled bovine serum albumin, BSA-FITC) was printed onto the copolymer coating using a BioOdyssey Calligrapher miniarrayer, and the conditions for protein printing — concentration, pH, temperature, and relative humidity (RH) — were optimized.

Methods

Surface Modification of the Glass Substrate

Microscope glass slides were cleaned by sonication in surfactant solution followed by soaking in NaOH solution and washing in ultrapure water. ALAPP coatings were deposited onto glass slides using a custom reactor. Copolymer of PEGMA/GMA prepared with different molar ratios was deposited onto glass slides using thermal initiator 2,2'-azobisisobutyronitrile (AIBN) by spin coating, dip coating, and in situ grafting. Bis (2-aminoethyl) polyethylene glycol 3400 was used as a crosslinker in the case of spin coating.

Microarray Printing

A BioOdyssey Calligrapher miniarrayer with temperature and humidity control was used for contact printing of BSA-FITC. An ArrayIt round solid pin with a tip diameter of 375 µm (TeleChem International) was used for printing. The following printing conditions were used: Pin approach speeds to the source plate and glass slide were 15 and 5 mm/sec, respectively; dwell times in source plate and on glass slide were 1,000 and 35 ms, respectively. Protein concentration, pH, temperature, and RH were varied to determine the optimal conditions for microarraying.

Glass slides were stored in the refrigerator for 10 hr after printing and subsequently washed and soaked in phosphate buffered saline (PBS), pH 7.4, at 37°C overnight. Slides were then washed with ultrapure water and dried by purging with dry nitrogen gas. Printed glass substrates were analyzed using a GenePix 4000A microarray scanner (Molecular Devices) at a resolution of 10 µm.

Results and Discussion

Proteins were first printed on the unmodified glass substrate to investigate whether the BSA-FITC bound to the bare glass surface. As shown in Figure 1A, the distribution of the protein was uniform within the spots. After overnight soaking of the glass slide in PBS, however, spots were completely washed off (Figure 1B), indicating that no chemical bonding occurred between the glass surface and the protein molecules.

Fig. 1. Binding of protein to unmodified glass substrate. Fluorescence images of BSA-FITC spots, before (A) and after (B) washing in PBS.

When preparing protein-reactive glass surfaces, producing a uniform coating is highly desirable to reduce the influence of surface inhomogeneities on the spot-to-spot variation in the microarray. Previous surface analyses of PEGMA/GMA-coated slides have shown that spin-coated slides have the most uniform surfaces, followed by dip-coated slides and, lastly, grafted slides (Kurkuri et al. in press, Thissen et al. in press). Hence, in this study, spin- and dip-coated slides were further characterized in terms of their performance as microarray substrate surfaces. It was expected that proteins would be covalently immobilized on the coated substrate surface through the reaction of protein amino groups within the oxirane ring of GMA.

To investigate the optimal protein concentration and pH at which uniform circular spots can be reproducibly obtained, four protein concentrations (200, 100, 75, and 50 µg/ml) were prepared in buffers at four different pH values (pH 5, 7, 9, and 10) and printed at 10°C and 65% RH on glass slides spin-coated with PEGMA/GMA copolymer. As shown in Figure 2, protein spots can clearly be seen even after soaking in PBS overnight. All protein concentrations produced spots with detectable fluorescence intensity. The most circular spot geometry was observed when spots were printed using pH 5 buffer. Spots printed at pH 7 were also circular, but spots printed at pH 9 or 10 had irregular outlines. On the other hand, the spots printed at pH 9 or 10 showed greater fluorescence levels than those printed at pH 5 or 7. Similar results were obtained with dip-coated slides (data not shown).

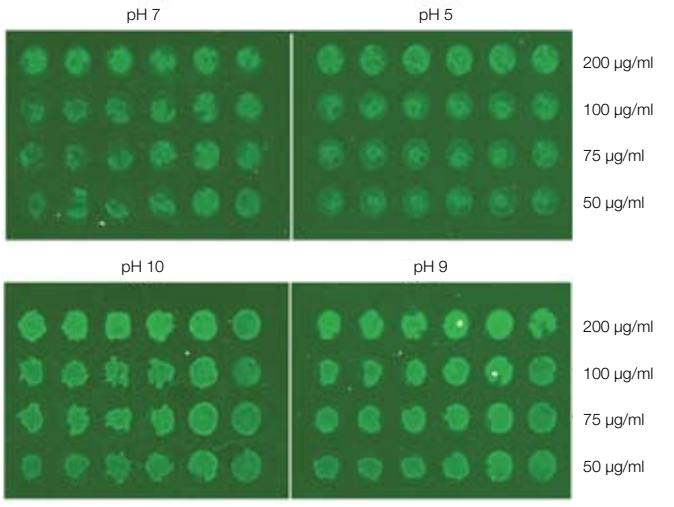

Fig. 2. Concentration and pH dependence of protein immobilization. Fluorescence images show arrays of BSA-FITC printed onto a glass slide that was spin-coated with PEGMA/GMA copolymer. Four different protein concentrations in buffer at four different pH values are shown.

Temperature and RH are known to play important roles in immobilization of proteins on microarray spots. The temperature at which the slide is held determines the kinetics of protein immobilization, while the RH influences the rate of evaporation of the nanoliter droplets. Therefore, experiments were performed to study the effects of temperature and RH on protein printing.

Images of dip-coated slides printed at different temperatures are shown in Figure 3. It is clear from the images that 10 and 15°C were ideal for printing biomolecules onto the reactive glass slides. In contrast, spots were incomplete when printing was performed at 5°C, presumably due to poor reaction kinetics. Printings obtained at temperatures of 10 and 15°C showed better protein attachment to the glass substrate. We selected 10°C for subsequent experiments in order to slow the evaporation of solvent from the protein solution, thus allowing the immobilization reaction to proceed from the liquid phase for a longer time. Binding to spin-coated slides showed similar temperature dependence (data not shown).

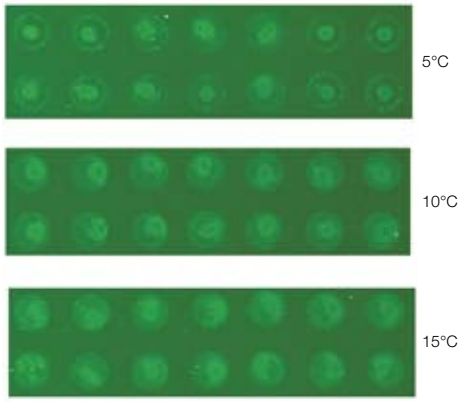

Fig. 3. Temperature dependence of protein immobilization. Fluorescence images show dip-coated glass slides printed with 75 µg/ml BSA-FITC in PBS, pH 5, at 65% RH. Three different temperatures are shown.

Figure 4 shows the effect of RH on protein attachment. Spots printed at 35, 45, or 55% RH were irregular in shape, and almost completely washed off. This may have been due to faster evaporation of solvent molecules from protein solution printed at lower RH. The spots printed at 65% RH remained attached even after soaking in PBS overnight, suggesting that higher RH is better for printing protein molecules onto reactive polymer coatings.

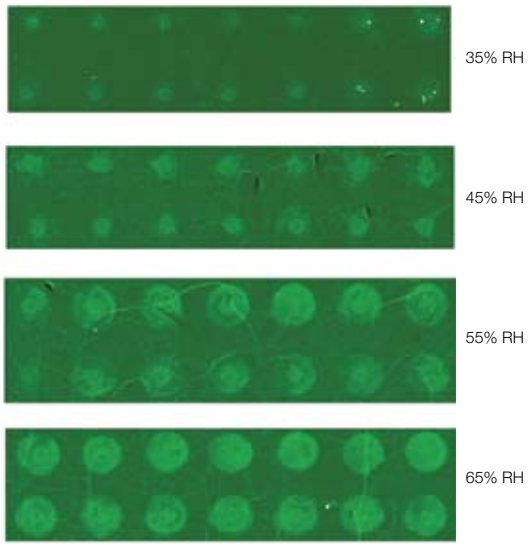

Fig. 4. The effect of RH on protein immobilization. Fluorescence images show 75 µg/ml BSA-FITC printed onto a dip-coated glass slide at 10°C and pH 5. Four different RH levels are shown.

Conclusions

PEGMA/GMA copolymer coatings were prepared on glass slides using three different coating methods: spin coating, dip coating, and surface grafting. The BioOdyssey Calligrapher miniarrayer was successfully used to array proteins onto the modified glass substrates. Optimal protein concentration, pH, temperature, and humidity conditions for protein printing were identified. The surfaces presented in this study are currently used in cell microarray experiments in our laboratories, and initial cell culture results have confirmed the ability to immobilize biologically active compounds and have shown the intended low levels of cell attachment to these coated slides.

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Monitoring Development of Chromatographic Methods With the Experion™ Automated Electrophoresis System

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Introduction

Gel electrophoresis is the standard method for evaluating results from chromatography experiments, but even with modular precast gel and buffer systems, a great deal of hands-on work, time, and laboratory space is required to obtain results. These limitations are compounded by the effort required to produce data in a format suitable for laboratory notebooks, meetings, presentations, and the electronic archival systems used by many companies to support their regulatory compliance programs. These challenges create serious bottlenecks for all protein chemists, but especially for process developers who perform many chromatography runs a day, with each run requiring time-consuming electrophoretic analysis of multiple fractions.

The Experion automated electrophoresis system and Pro260 analysis kit can be used to accelerate the process of developing a purification scheme for an antibody or other protein. The Experion system combines electrophoresis, staining, destaining, detection, basic analysis, and digital result documentation into a single automated step, allowing analysis of up to ten protein samples in as little as 30 min. The Experion system provides rapid analysis of column fractions, including protein sizing, quantitation, and purity information, enabling the scientist to make accurate decisions regarding the purification steps in real time. Data can be shared immediately in presentations, analysis, or discussion, which enables process developers to quickly move on to the next phase of experimentation. Moreover, with the validated US FDA 21 CFR Part 11-compliant features of the Experion Security Edition software, the electronic data can be easily archived or submitted to the FDA.

In this technical report we examine the data generated in a 3-day workshop to illustrate how the Experion system fits into the process development workflow. During the workshop, students were divided into teams and provided with a murine IgG_1 monoclonal antibody (MAb) previously purified over a protein A affinity resin. Their task was to identify and optimize an appropriate chromatographic purification method using hydrophobic interaction, ion exchange, and/or ceramic hydroxyapatite media. We present some of the results from one group that developed a purification process using the BioLogic DuoFlow™ chromatography system and CHT™ ceramic hydroxyapatite Type I support. The Experion system was used to evaluate antibody purity and yield in various column fractions.

Methods

Antibody Purification

Workshop participants were provided with a murine IgG_1 MAb that had been isolated from a cell culture supernatant derived from cell line AE-1 (Invitrogen Corp.). The MAb had been purified as follows: A 5 ml HiTrap column connected in series with a MabSelect SuRe protein A column (both from GE Healthcare) was equilibrated with 50 mM Tris, 4 M NaCl at pH 9. The MAb-containing sample was titrated to pH 6.5 with 0.5 M NaH_2PO_4 and loaded onto the column. The antibody was eluted with 0.1 M glycine, 50 mM NaCl, pH 3 and then neutralized with 1 M Tris, pH 9 and 10 mM NaH_2PO_4.

Participants then applied the protein A-purified MAb over a CHT ceramic hydroxyapatite, Type I, 20 µm column equilibrated with 10 mM NaH_2PO_4, pH 6.5. The column was washed with 2 column volumes of 10 mM NaH_2PO_4, and the antibody eluted using a linear salt gradient to 10 mM NaH_2PO_4, 1 M NaCl, pH 6.5. To check the quality of the purified product, size exclusion chromatography was then performed on a 24 ml Superdex 200 column (GE Healthcare) in 2x PBS containing 2 M urea, pH 7.2. All separations were performed on a BioLogic DuoFlow system.

Experion Pro260 Analysis

Experion Pro260 analysis kits include Experion Pro260 protein ladder, sample buffer, gel solution, fluorescent stain, spin filters, and microfluidic chips. Reducing and nonreducing sample buffers were prepared by mixing 30 µl of Experion Pro260 sample buffer stock either with 1 µl of β-mercaptoethanol or with 1 µl of deionized water, respectively. Antibody samples were prepared under both reducing and nonreducing conditions

by mixing 4 µl of sample with 2 µl of the appropriate sample buffer, heating for 5 min at 95°C, and then diluting with 84 µl of 0.2 µm filtered water. Prepared samples were then loaded onto chips that were primed according to the protocol described in the Pro260 analysis kit instruction manual. Initial MAb samples (protein A-purified) required dilution in 1x PBS buffer prior to Experion analysis, while samples taken at various time points during CHT purification were used directly, without dilution.

Results and Discussion

Development of process- and research-scale MAb purification procedures can be significantly accelerated with online detection tools for quantitation and qualification of samples. In this tech note we demonstrate how use of the Experion system can accelerate workflow and process development.

A murine IgG_1 MAb was purified in a two-step process using protein A and ceramic hydroxyapatite media. At each step of the purification scheme, a sample was run on an Experion Pro260 protein chip. Experion software generated an electropherogram, simulated gel image, and results table for each sample.

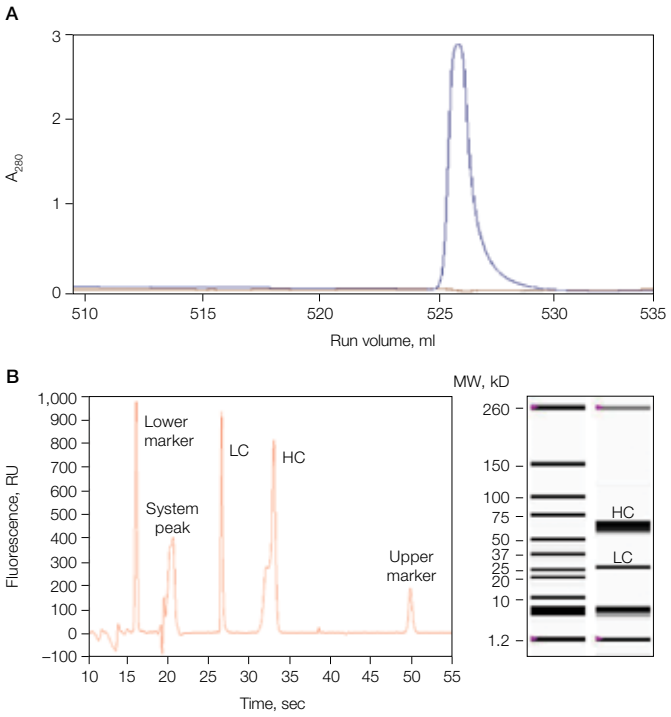

Fig. 1. Protein A purification of murine IgG_1 MAb. A, chromatogram generated by BioLogic DuoFlow software, showing the protein A-captured monoclonal IgG; **B**, Experion Pro260 analysis showing the electropherogram (left) and virtual gel image (right) of the eluted protein fraction under reducing conditions. Left lane of gel image, Pro260 protein ladder; right lane, MAb-containing fraction.

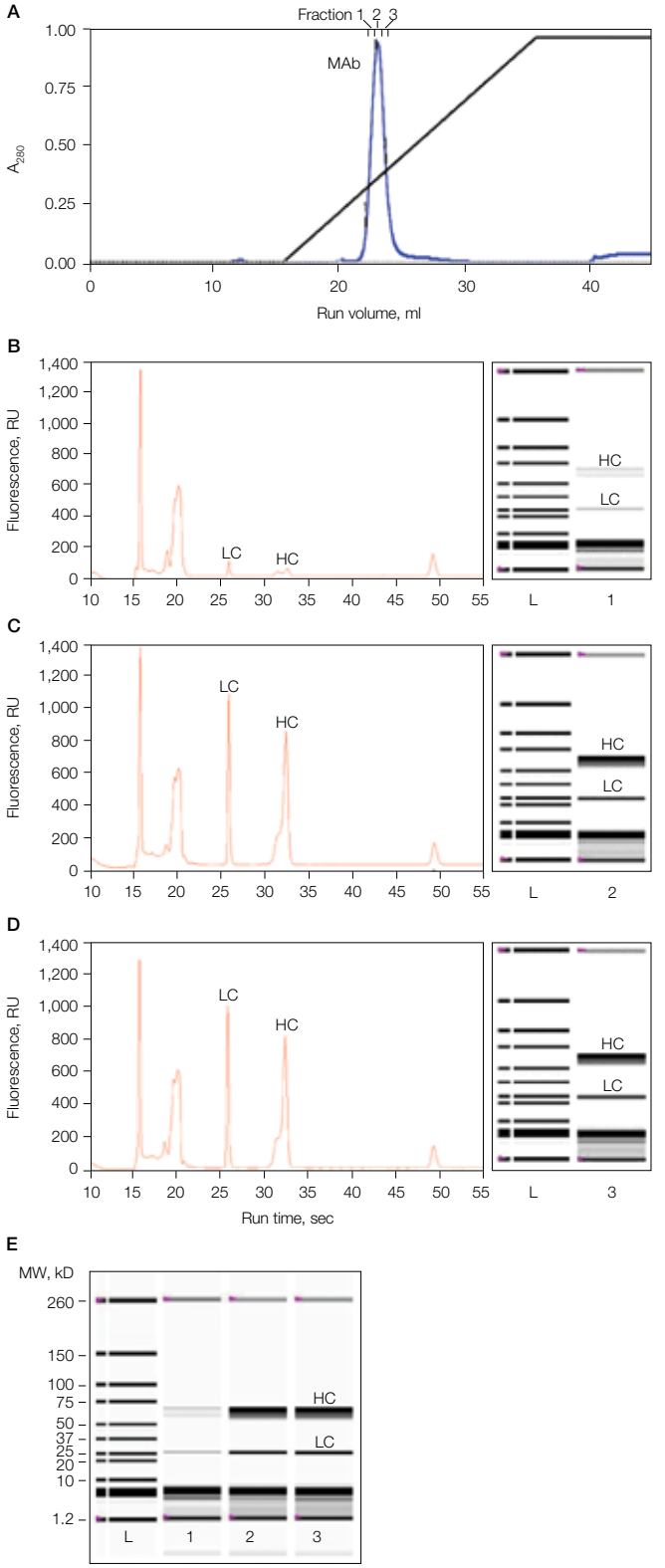

Fig. 2. CHT purification of protein A-purified murine IgG_1 MAb. A, chromatogram generated by BioLogic DuoFlow software showing the CHT-purified antibody. Three fractions, designated 1, 2, and 3, were chosen for Experion analysis. Blue trace, A_{280}; black trace, NaCl gradient. **B–D**, Experion Pro260 analysis showing the electropherograms (left) and virtual gel images (right) of the eluted protein fractions 1 (B), 2 (C), and 3 (D). Left lane of gel image, Pro260 protein ladder; right lane, MAb-containing fraction. **E**, composite gel image comparing all three fractions.

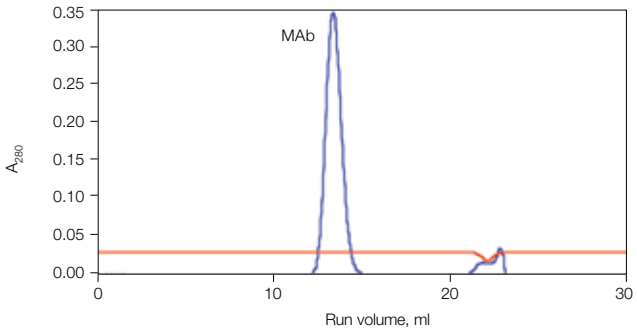

Fig. 3. Size exclusion chromatography of purified protein. Note the symmetrical protein peak, which indicates that the CHT-purified MAb is free of contaminants and aggregates.

The MAb was first captured on a MabSelect SuRe protein A column (Figure 1A). A sample of the eluted peak was analyzed with the Experion system under reducing conditions and showed peaks at 27 kD and 66 kD, representing the light chain (LC) and heavy chain (HC) of the antibody (Figure 1B). The heavy-chain peak showed broadening at 60 kD, which was possibly the result of the different glycosylation states of the heavy-chain protein.

The protein A-captured antibody was then passed over a CHT ceramic hydroxyapatite column to further purify the MAb and to remove antibody aggregates, leached protein A, DNA, and lipopolysaccharides (Figure 2A). Three fractions taken during elution of the peak were analyzed by the Experion system, also under reducing conditions, and showed peaks at 25 kD and 64 kD, representing the light chain (LC) and heavy chain (HC) of the antibody (Figure 2B–E). The Experion analysis, from sample preparation through the chip run, was achieved in 30 min, much faster than the 2–3 hr generally required for SDS-PAGE.

As a further method of quality control, a sample of the CHT-purified MAb was analyzed by size exclusion chromatography (Figure 3), which showed a symmetrical peak, indicating that the MAb was free of contaminants and aggregates.

Conclusions

Experion Pro260 analysis is comparable to traditional SDS-PAGE in terms of performance (Chang et al. 2005, Zhu et al. 2005) but is a more rapid analysis that complements the need for quick data acquisition during process development. Experion analysis, from sample preparation through the chip run, is achieved in as little as 30 min compared to the 2–3 hr generally required for SDS-PAGE. Experion software automatically calculates the molecular weight of each resolved protein in a sample and provides information regarding the relative sample concentration and purity of each sample. The optional Security Edition software offers tools for compliance with US FDA 21 CFR Part 11 regulations.

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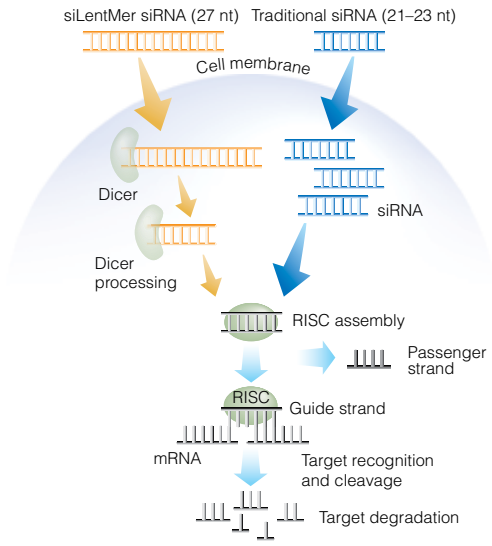

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